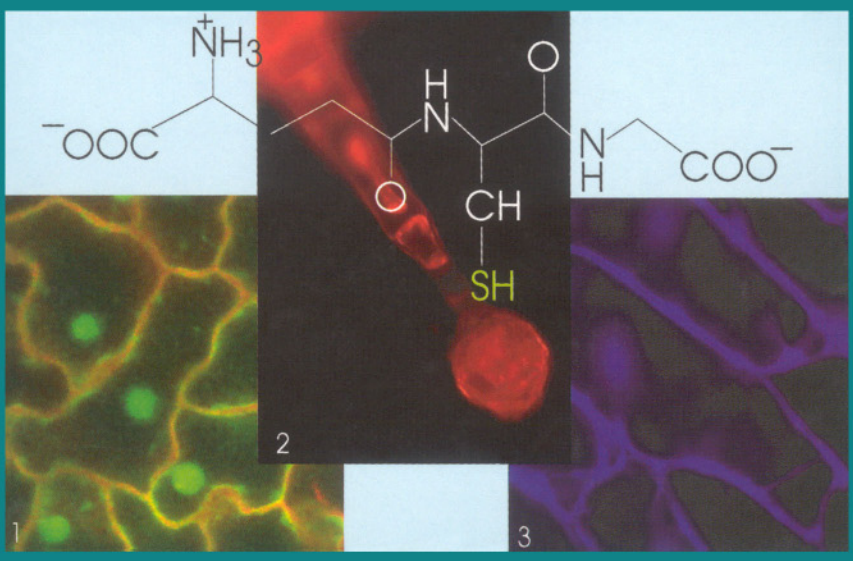
PLANT ECOPHYSIOLOGY

Significance of Glutathione in Plant Adaptation to the Environment

Edited by
Dieter Grill
Michael Tausz
and Luit J. De Kok



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SIGNIFICANCE OF GLUTATHIONE TO PLANT ADAPTATION TO THE ENVIRONMENT

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The Kluwer Handbook Series of Plant Ecophysiology comprises a series of books that deals with the impact of biotic and abiotic factors on plant functioning and physiological adaptation to the environment. The aim of the Plant Ecophysiology series is to review and integrate the present knowledge on the impact of the environment on plant functioning and adaptation at various levels of integration: from the molecular, biochemical, physiological to a whole plant level. This Handbook series is of interest to scientists who like to be informed of new developments and insights in plant ecophysiology, and can be used as advanced textbooks for biology students.

The titles published in this series are listed at the end of this volume.

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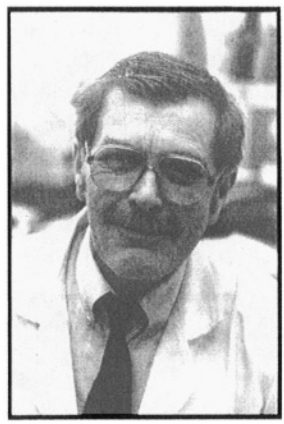
Dedicated to our friend, colleague and teacher
Prof. Dr. Hermann Esterbauer who died too early



Prof. Dr. Hermann Esterbauer,
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1936 – 1997

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Contents

Preface

The present volume of Plant Ecophysiology focuses on the biological significance of glutathione in plants. Glutathione or its homologues are present in nearly all living organisms and are directly or indirectly involved in many important metabolic reactions and in physiological functioning. The current ideas on the role of glutathione in plant metabolism and its significance in plant adaptation to biotic and abiotic stress is discussed in the different chapters of the volume. Occasional overlaps of information between chapters could not be avoided; moreover, they reflect the central and multiple role of glutathione in integrated plant metabolism.

The main aim of this volume is to raise the interest of advanced students and junior researchers in the role of glutathione in plants and to supply basic and comprehensive information for scientists already working on related topics. It must be emphasized that it was not the intention of the authors to present detailed scientific reviews about the various aspects of glutathione for specialists.

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Chapter 1

GLUTATHIONE – AN ANCIENT METABOLITE WITH MODERN TASKS

Heinz Rennenberg

INTRODUCTION

Glutathione and its homologues comprise a group of tripeptides that are synthesised from the constituent amino acids rather than by direct translation of mRNA. The group of tripeptides is characterised by an N-terminal γ-glutamyl moiety, a central cysteine residue, and a variable C-terminal amino acid. Numerous functions have been attributed to glutathione and its homologues that are all connected with its cysteine residue. These functions include storage and transport of reduced sulphur (Herschbach and Rennenberg 1997, 2001), regulation of sulphur nutrition (Herschbach and Rennenberg 1997, 2001), compensation of oxidative stress as chemical anti-oxidant and co-substrate in enzymatic reactions (Noctor and Foyer 1998), redox regulation and buffering (Kunert and Foyer 1993), regulation of enzyme activity, mRNA translation and gene transcription (Fahey et al. 1982, Kunert and Foyer 1993, Foyer and Rennenberg 2000), modification and transport of hormones, and detoxification and transport of xenobiotics (Marrs 1996) or heavy metals (Zenk 1996, Rauser 1999). Many of these functions are connected with particular developmental stages and/or environmental conditions; they are often restricted to specific tissues and/or sub-cellular compartments. Still it appears that, even in a particular sub-cellular compartment of plant cells, several functions have to be fulfilled simultaneously by glutathione. The regulatory processes that enable a concerted action of glutathione in different functions within and beyond sub-cellular compartments are virtually unknown. Even at the cellular level, basic responses of glutathione to developmental and environmental changes are not understood, because analyses have been carried out only at the plant organ rather than the cellular level. In this respect, recent advances in plant molecular biology and in *in vivo* imaging techniques have opened an exciting new field of research.

In the present report, a personal view of the development of research on glutathione in plant sciences is provided and the significance of glutathione

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as an essential metabolite of primary metabolism is discussed. The partitioning of glutathione within plants and its allocation are characterised in view of the functions of glutathione in whole-plant sulphur nutrition.

STUDIES ON GLUTATHIONE IN PLANTS: A HISTORICAL VIEW

Glutathione was first detected in plant cells. In 1888, De Rey-Pailhade found a compound in yeast and other cells that spontaneously reacted with elemental sulphur to yield hydrogen sulphide. From the Greek words for love and sulphur he chose the name "philothion" for his new substance (see: Meister 1988). Subsequent studies led Hopkins to the conclusion that this compound was a dipeptide containing cysteine and that a compound of glutamate and γ-glutamylcysteine was the most probable structure (see: Meister 1988). The name "glutathione" was chosen by Hopkins, because of its link with the historic philothion, the termination of "peptone" as a name for small peptides, and a reminder that it contains glutamic acid linked to a sulphur compound. In a reinvestigation in 1929, Hopkins found that the presence of glycine had been overlooked in his former work and in 1930 the structure of glutathione was identified as L-γ-glutamyl-L-cysteinyl-glycine (see: Meister 1988). This structure was frequently confirmed in subsequent studies by chemical and enzymatic synthesis, degradation, and physical methods.

In the subsequent decades research on glutathione in mammalian biochemistry and medical sciences rapidly evolved, but in plant sciences glutathione was largely overlooked. In 1957, Price observed a compound in legumes with properties similar to glutathione and named this compound "phaseqthione" (Price 1957). Seven years later this compound was isolated, identified, as L-γ-glutamyl-L-cysteinyl-β-alanine, and renamed homoglutathione (Carnegie 1963a,b). Glutathione *S*-transferase (GST) enzymes in plants were discovered in maize and related species with C_4 photosynthesis in 1970 (Frear and Swanson 1970). From this discovery a whole new area of research on glutathione with strong emphasis on practical application for agriculture developed. In the late 1970s and early 1980s several major advances in plant research on glutathione were achieved. Glutathione and glutathione reductase were identified in the chloroplasts and its role in ascorbate metabolism was suggested in 1976 (Foyer and Halliwell 1976). From this finding the idea of compensation of oxidative stress by glutathione developed. In the same year it was shown that cultured tobacco cells release glutathione into the culture medium (Rennenberg 1976) and it was suggested that glutathione could be a long-distance transport form of reduced sulphur, a function of glutathione first demonstrated in experiments with tobacco

plants in 1979 (Rennenberg et al. 1979). Seasonal storage of glutathione was found in spruce needles by Esterbauer and Grill (1976). Four years later Fahey et al. (1982) showed that protein synthesis in the wheat embryo is regulated by the state of oxidation of glutathione and indicated a role of oxidized glutathione (GSSG) as inhibitor of ribosomal functioning in plants. This discovery may be considered a starting point of plant molecular biology in research on glutathione that began to proliferate about 10 years later.

The first comprehensive review on glutathione in plants was published by Rennenberg (1982). At this time, already a large number of books on glutathione were being published in biochemistry and medical sciences, but glutathione was still not a recognized topic in plant biology. Only a few years later the number of publications on glutathione in plants virtually began to explode, when it became obvious that glutathione was a central component of the plants defence system against various forms of natural and man-made stresses (Rennenberg and Brunold 1994, May et al. 1998). During this time, phytochelatins were also identified as glutathione derivatives that are able to bind heavy metals (Grill et al. 1985) and can contribute to heavy metal homeostasis in plants living in contaminated environments (Grill et al. 1988). Initially, the approaches to connect glutathione levels with stress tolerance were often not satisfying, because the results from laboratory and field studies that provided circumstantial evidence, partially were contradictory and causal relationships were difficult to establish with the methods applied. This situation improved when methods of plant molecular biology became available in the early 1990s. In the meantime, genetic engineering has enhanced considerably the understanding of the regulation of glutathione synthesis and its significance for plant performance under stressful conditions (May et al. 1998, Noctor and Foyer 1998, Noctor et al. 1998). Much of this progress became possible, when the genes encoding the enzymes of glutathione synthesis, *gshI* and *gshII*, were isolated and sequenced from *E. coli* (Gushima et al. 1984, Watanabe et al. 1986), and were successfully used for the transformation of higher plants (Noctor et al. 1998, Foyer and Rennenberg 2000).

There was a price to be paid for concentrating on regulatory aspects of stress physiology and plant molecular biology of glutathione in the 1990s. In the early 1990s, new homologues of glutathione were identified, probably more by accident than through specific surveys for such compounds. Hydroxymethyl-glutathione was found in addition to glutathione in the Poaceae (Klapheck et al. 1992), and γ-glutamylcysteinylglutamic acid an cadmium exposed maize plants (Meuwly et al. 1993). These findings strongly suggested a considerable heterogeneity in glutathione homologues with different C-terminal amino acids in the plant kingdom. Unfortunately, these findings did not result in a directed approach to characterize the biodiversity of glu-

tathione derivatives in plants. Therefore, when starting to work with a new species, we have to face the fact that most analytical methods frequently used for quantification of glutathione do not *per se* distinguish between glutathione and its homologues and interpretation of results may be hampered by improper biochemical analysis. In addition, the reasons why glutathione homologues have evolved in the plant kingdom will remain unknown until comprehensive research is devoted to this particular aspect of research.

OCCURRENCE OF GLUTATHIONE: EVOLUTIONARY ASPECTS

Glutathione and/or its homologues are the most abundant low molecular weight thiols in most animal and plant cells (Meister 1975, Kasai and Larson 1980, Klapheck 1988, Klapheck et al. 1992, Meuwly et al. 1993). High concentrations of thiols have also been determined in prokaryotes, but a number of these organisms, in particular anaerobic bacteria, contain low concentrations of glutathione (Fahey et al. 1978). In some of these bacteria, co-enzyme A or the metabolic glutathione precursor γ-glutamylcysteine are the dominant low molecular weight thiols (Newton and Javor 1985, Setlow and Setlow 1977). It may therefore be concluded that glutathione evolved in an anaerobic environment, but became essential for life when oxygen containing environments developed. The selective disadvantage of co-enzyme A or γ-glutamylcysteine as a replacement for glutathione in the presence of oxygen remains to be elucidated. An essential requirement of glutathione for plants living in an oxygen-containing atmosphere is also indicated by observations with the *rml1* mutant of *Arabidopsis.* This mutant does not contain measurable amounts of glutathione, due to its deficiency in γ-glutamylcysteine synthetase, and as a consequence exhibits a particular phenotype with lacking root development and a small shoot; it can survive only in tissue culture when supplemented with glutathione in the culture solution (May et al. 1998). Mutants of *E. coli* that lack the enzymes of glutathione synthesis and, therefore, appreciable glutathione contents are highly susceptible to oxygen, and to a wide range of chemical agents. In the absence of oxygen and adverse chemical agents they grow at the same rate as the parental strain (Apontoweil and Behrends 1975). Thus it is most likely that evolution of glutathione was not only an essential prerequisite for survival in an oxygen-containing environment, but also for the defence against various environmental stresses. These functions of glutathione are also obvious in present-day plants.

From their distribution in the plant kingdom, glutathione homologues such as homo-glutathione or hydroxymethyl-glutathione appear to be recent

inventions of evolution. However, the evolutionary advantage of these inventions is obscure, since numerous studies indicate that glutathione and its homologues appear to fulfil similar functions in plant cells (Bergmann and Rennenberg 1993).

DISTRIBUTION OF GLUTATHIONE WITHIN PLANTS

Glutathione is a constituent of all plant organs, but its concentration differs considerably between organs and within the same organ at different developmental stages and under different environmental conditions. For example, glutathione concentrations are usually lower in the roots compared to leaves, and higher in young developing than in mature leaves (Klapheck 1988, Arisi et al. 1997, Hartmann et al. 2000, Herschbach et al. 2000). In conifer needles, glutathione concentrations are high in winter and low in summer (Esterbauer and Grill 1976, Schupp and Rennenberg 1992). Low concentrations of glutathione during summer were also observed in the leaves of deciduous trees (Polle et al. 1992). Particularly high glutathione concentrations have been determined in seeds (Klapheck 1988). Concentrations of glutathione in the phloem were similar to those determined in leaves and did not change considerably during long-distance transport from the leaves to the roots (Rennenberg 1999, Foyer and Rennenberg 2000). Glutathione has also been determined in the xylem sap of numerous tree species (Herschbach and Rennenberg 1997, Rennenberg 1999), but often cysteine exceeded the glutathione content (Rennenberg et al. 1994a). Glutathione concentrations in roots and xylem were generally lower than in leaves or phloem. In the xylem sap of mature beech trees, glutathione concentrations strongly depended on the season, but did not change considerably along the transport path (Schupp et al. 1991, Rennenberg et al. 1994b). Glutathione contents in the xylem sap of oak seedlings and young poplar trees declined by about one order of magnitude from the roots to the shoots, indicating that glutathione is removed from the xylem during its long-distance transport (Rennenberg 1999, Foyer and Rennenberg 2000). Whether these differences in downloading of glutathione from the xylem depend on plant age or the species analysed remains to be elucidated. Downloading of glutathione during long-distance transport in the xylem also explains the observation that glutathione is frequently found in the xylem, but is virtually absent from the apoplastic space of leaves (Polle et al. 1990, Vanacker et al. 1998).

Environmental factors such as sulphur or nitrogen nutrition (Rennenberg 1984, De Kok 1990, Brunold 1993) and various stress conditions (Rennenberg and Brunold 1994, May et al. 1998) affect glutathione levels in different ways. Whereas excess sulphur in the environment elevates glutathione

levels, the consequences of different stresses on glutathione levels are often inconclusive, as enhanced, reduced and unchanged levels have been reported in different studies. Part of these contradictions result from insufficient characterization of "plant history" and environmental conditions. For example, mycorrhization was found to enhance the glutathione content in both phloem and xylem of oak *(Quercus robur* L.) seedlings (Rennenberg 1999). Although it is uncertain whether or not this observation can be extrapolated directly to other species, it may explain inconsistencies between the results of laboratory studies, usually carried out with non-mycorrhizal plants, and studies in the field, where most plants live in association with mycorrhizal fungi.

Presently, quantitative information on glutathione concentrations of individual cell types of intact plants is not available. However, recent technologies . of *in vivo* glutathione analysis by laser scanning microscopy (Fig. 1 cover page) will provide such information in the near future (Meyer and Fricker 2000). The application of this technology has already suggested remarkable differences in glutathione contents of different cell types *in Arabidopsis* roots (Sánchez-Fernández et al. 1997). Similarly, relatively few data have been published on the sub-cellular distribution of glutathione. Based on total cellular glutathione concentrations and the relative volume of chloroplasts, cytosolic concentrations were estimated to be in the region of 0.1-0.2 mM, while chloroplast concentrations were in the region of 2-4 mM (Rennenberg 1982). In these studies an equal distribution of glutathione between the cytosol and the vacuole was assumed. However, since numerous experiments aiming to detect glutathione in vacuoles have failed, it issmore likely that the presence of glutathione is restricted to the cytoplasm and that cytosolic glutathione concentrations are similar to those reported for chloroplasts.

THE ROLE OF GLUTATHIONE IN WHOLE-PLANT PHYSIOLOGY OF SULPHUR NUTRITION

In order to maintain appropriate sulphur nutrition at the whole plant level, sulphate uptake and sulphate reduction have to be regulated in order to satisfy the sulphur demand of the shoot and roots. Therefore, it has been suggested that allocation of a regulatory signal from the shoot to the roots is involved in sulphur nutrition (Rennenberg 1984, 1995). Glutathione was suggested to constitute such a signal, because it (1) reduced sulphate uptake in heterotrophic, but not in photoheterotrophic tobacco cell cultures (Rennenberg et al. 1988) and (2) is the predominant thiol allocated from mature tobacco leaves to the roots (Rennenberg et al. 1979). Experiments on glu-

tathione transport from the shoot to the roots and its influence on sulphate uptake and sulphate transport to the shoot support this hypothesis. Feeding glutathione to mature tobacco leaves diminished sulphate uptake by the roots up to 46 % (Herschbach and Rennenberg 1994). As a consequence of H_2S and SO_2 exposure of spinach leaves, thiol contents of the roots increased (Herschbach et al. 1995a) and sulphate uptake and/or sulphate transport to the shoot were reduced in tobacco and spinach depending on sulphur nutrition (Herschbach et al. 1995a,b). A similar regulation of nitrate uptake by phloem allocated glutamine was observed as a consequence of ammonia fumigation of beech trees (Geßler et al. 1998).

In a split root experiment, Lappartient and Touraine (1996) demonstrated directly that glutathione can act as a phloem-allocated signal, which regulates sulphate uptake in *Brassica napus.* In this experiment part of the root system was subjected to sulphur starvation, whereas the remaining part was exposed to sulphur sufficient conditions. When glutathione was fed to one part of the roots in the split root system, sulphate uptake by the other part was inhibited (Lappartient and Touraine 1996). Since glutathione levels decreased in phloem exudates after sulphur starvation and increased after feeding glutathione to sulphur-sufficient roots, the authors concluded that glutathione is the inter-organ signal regulating sulphate uptake. With the same split root system Lappartient et al. (1999) showed enhanced transcript levels of the low affinity sulphate transporter (AST68) in the sulphur sufficient part of the roots of *Brassica napus* and *Arabidopsis thaliana,* when the other part was starved for sulphate. Enhanced transcript levels were accompanied by low glutathione, but not by low cysteine or sulphate contents in phloem exudates (Lappartient and Touraine 1996). Conversely, the low transcript level of sulphate transporter in the roots of sulphur sufficient plants was attributed to the additional glutathione transported in the phloem (Lappartient et al. 1999).

Regulation of sulphur nutrition to sulphur demand in poplar plants is found to be more complex. Transgenic poplars over-expressing γ-glutamylcysteine synthetase in the cytosol contain higher cysteine, γ-glutamylcysteine, glutathione and sulphate contents in phloem exudates compared to wildtype plants (Herschbach et al. 1998, 2000). Since glutathione is thought to repress sulphate uptake in many plants (Herschbach and Rennenberg 1991, 1994, Herschbach et al. 1995a,b, Lappartient and Touraine 1996, Lappartient et al. 1999), down-regulation of sulphate uptake must be expected under these conditions. On the other hand enhanced glutathione synthesis by the transgenic plants will require additional sulphate and, hence, elevated sulphate uptake. Experiments with detached roots indeed showed enhanced sulphate uptake by the roots in transgenic compared to wildtype poplar, irrespective of the elevated glutathione level (Herschbach

et al. 2000). This result clearly demonstrates that a high glutathione transport in the phloem does not necessarily cause reduced sulphate uptake by the roots. Increased sulphate uptake by the roots of transgenic poplar plants was accompanied by enhanced sulphate contents in the phloem, also demonstrating that sulphate could not be the phloem-mobile signal reducing sulphate uptake by the roots (Herschbach et al. 2000). Thus, neither phloem-allocated glutathione nor phloem-allocated sulphate seems to regulate sulphate uptake by the roots of poplar under conditions of enhanced demand.

Under conditions of decreased sulphur demand, mediated by H_2S fumigation of the leaves, enhanced glutathione transport in the phloem reduced sulphate transport to the shoots of poplar plants; it increased glutathione contents in phloem exudates and reduced sulphate transport to the shoot (Herschbach et al. 2000). Correlation analysis revealed that the sulphate-to-glutathione ratio in the phloem might be the inter-organ signal regulating sulphate uptake and xylem loading of sulphate (Herschbach et al. 2000). High sulphate-to-glutathione ratios in the phloem seem to signal that excessive sulphur nutrition and sulphate uptake, as well as sulphate transport to the shoot are diminished. Low sulphate-to-glutathione ratios in the phloem mediate increased sulphate uptake by the roots and increased sulphate transport to the shoot. Therefore, it may be assumed that the sulphate-to-glutathione ratio in the phloem regulates sulphur nutrition at the whole plant level.

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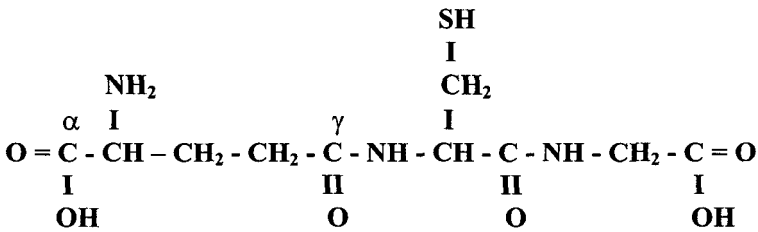
Chapter 2

CHEMISTRY OF GLUTATHIONE

Willibald Wonisch and Rudolf J. Schaur

The reduced form of glutathione (GSH)



OCCURRENCE AND FUNCTION

The ubiquitous tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) occurs in most plants and bacteria as well as in animals in concentrations ranging from 0.1 - 10mM (Meister and Anderson 1983), e.g. in spinach chloroplasts a concentration as high as 3 mM has been observed. It is possibly the most abundant redox scavenging molecule in cells and its concentration is in a highly complex manner dependent on the carbon source and varies as a function of age, as is also the case in microorganisms, animals and human (Lamoureux and Rusness 1989), e.g. stationary phase cells showing a high glutathione content whereas logarithmic growth is associated with low levels of GSH (Berthe-Corti et al. 1992).

The intracellular glutathione (e.g. in tobacco mesophyll cells) is distributed as follows: chloroplasts 76% vacuoles 17% cytoplasm 7%.

Some leguminous species e.g. soybean (*Glycine max*), white clover (*Trifolium repens*) or lima bean (*Phaseolus limensis*) contain homoglutathione (γ-glutamylcysteinyl-β-alanine) instead of glutathione as the major form of free thiol, whereas in pea (*Pisum sativum*) and peanut (*Arachis hypogea*) glutathione is the major primary thiol. Homoglutathione functions in a similar manner as glutathione as oxidized homoglutathione is also reduced by

D. Grill et al. (eds.), Significance of Glutathione to Plant Adaptation to the Environment, 13-26.

NADPH in the presence of glutathione reductase and it is also utilized as a substrate by glutathione *S*-transferases.

The intracellular GSH may be particularly critical in cellular organelles and organs exposed to huge amounts of free radicals, e.g. chloroplasts, vacuoles and cytoplasm in plants and lung and intestine in mammals.

GSH is the most prevalent intracellular thiol, which functions directly or indirectly in many important biological phenomena:

It participates in the reduction of disulphides and other molecules. It conjugates with compounds of exogenous and endogenous origin. GSH protects cells against the effects of free radicals as well as of reactive oxygen intermediates (Meister 1983) such as hydrogen peroxide and organic peroxides. Moreover, GSH is a coenzyme for several enzymes. It is necessary for the synthesis of proteins and deoxyribonucleotide precursors of DNA. The cellular turnover of glutathione is associated with its transport out of the cell, in its reduced form GSH. The generality of GSH transport implies this process being a protective mechanism for cell membranes, since there exists no extracellular mechanism for reduction of oxidized glutathione (GSSG). Therefore, GSH must be continuously supplied from the cell.

SOME PHYSICO-CHEMICAL PROPERTIES OF GLUTATHIONE

Glutathione as a tripeptide

Both GSH and GSSG are white, crystalline solids (melting points 192-195°C and 178-182°C, respectively; molecular weights 307.33 and 612.63), which dissolve easily in water,

Glutathione is provided with several hydrophilic groups: two carboxylic acid groups, one amino group, one thiol group, and two peptide bonds. The most important chemically reactive group with respect to its biological and biochemical activity is the thiol group. The combination of many hydrophilic functional groups with a low molecular weight renders GSH as well as GSSG highly water-soluble. In contrast cystine, the disulphide derived from cysteine, is biologically unsuitable for a thiol-disulphide system due to its relatively low water solubility. The γ-glutamyl linkage of GSH leads to an increase of reactivity with respect to the participation in the γ-glutamyl cycle and protects GSH against the attack of aminopeptidases.

The following proton dissociation constants for GSH and GSSG have been reported:

pK values of GSH: SH, 9.2; NH_3^+, 8.66; COOH, 3.53; COOH, 2.12.

pK values of GSSG: COOH, 2.6; COOH, 3.4; NH_3^+, 8.9-9.6

Thus the functional thiol group of GSH is at physiological pH nearly entirely in the undissociated form.

The standard redox potential E_o' of the pair GSH/GSSG at pH 7.0 amounts to –0.27 volts, which is similar to the value of the pair cysteine/cystine (-0.34 volts), but glutathione is provided with strongly reduced toxicity compared to cysteine.

Table 1. Bond lengths for molecules related to glutathione.

Bond	Length	Notes
C-C	1.53	C-COOH
C-C	1.51	
C-S	1.811	
C-H	1.09	$-NH_2$-C
S-H	1.33	
C-N	1.487	
C-N	1.325	CONH
C-N	1.455	CONH-C
C=O	1.24	CONH
C=O	1.25	COOH
N-H	1.07	$-NH_2$
N-H	1.04	CONH

Chemical bonds

Glutathione is not equipped with structural features that might lead to unusual bond lengths, bond angles, or bond energies. This is also the case for the disulphide apart from the dihedral angles through the sulphur-sulphur bond, which is the critical geometric parameter for GSSG. The extended form of glutathione is 15 Å long and the corresponding form of GSSG is approximately 23 Å long.

THE γ-GLUTAMYL CYCLE AND OTHER GSH-RELATED REACTIONS

The γ-glutamyl cycle

The synthesis of glutathione in plants occurs in the chloroplast and occurs by the same series of reactions as utilized in mammals which belong to the

so-called γ-glutamyl cycle (Figure 1; Meister and Anderson 1983, Foyer and Noctor; Anderson this volume).

Glutathione is synthesized *de novo* in two mRNA-independent steps which are catalysed by the consecutive action of the enzymes γ-glutamylcysteine synthetase (Griffith and Mulcahy 1999; Figure 1, reaction 1) and gluthathione synthetase (reaction 2). In the first step an amide bond is formed between the γ-carboxy group of glutamate and the amino group of cysteine at the expense of ATP. In the second step, again with consumption of ATP, glutathione synthetase catalyses the formation of a peptide bond between the carboxy group of cysteine and the amino group of glycine. Cysteine is usually the limiting substrate and γ-glutamylcysteine synthetase is allosterically feedback-inhibited by GSH (K_i about 1.5 mM).

The breakdown of GSH, GSSG and *S*-substituted glutathione is catalysed by γ-glutamyl transpeptidase, which transfers the γ-glutamyl moiety to acceptors such as amino acids, certain dipeptides, water and GSH itself (Figure 1, reaction 3). Cysteine is one of the most active acceptor amino acids.

In the course of glutathione transport across cell membranes GSH reacts with extracellular amino acids catalysed by γ-glutamyl transpeptidase to produce γ-glutamyl amino acids which are transported into cells. Intracellular γ-glutamyl amino acids are substrates of γ-glutamyl cyclotransferase (Figure 1, reaction 4) (in contrast to glutathione), which converts these compounds into the corresponding amino acids and 5-oxo-L-proline. The conversion of 5-oxo-L-proline to L-glutamate is an ATP-dependent step, which is catalysed by the intracellular enzyme 5-oxo-prolinase (Figure 1, reaction 5). The by-product cysteinylglycine formed in the transpeptidase reaction is split in the last step of the γ-glutamyl cycle by dipeptidase into the two amino acids, which can serve again as precursors for the synthesis of GSH (Figure 1, reaction 6).

S-substituted glutathione derivatives are produced by the spontaneous or glutathione *S*-transferases catalysed reaction of GSH with electrophilic compounds (Figure 1, reaction 7). This reaction plays an important role in the metabolism of xenobiotics.

In the metabolism of these *S*-substituted glutathione derivatives are two enzymes of the cycle involved as well. The γ-glutamyl moiety of such conjugates is removed by γ-glutamyl transpeptidase (Figure 1, reaction 3]), which facilitates the formation of γ-glutamyl amino acids. *S*-substituted cysteinylglycines are cleaved by dipeptidase (reaction 6a) to yield the corresponding *S*-substituted cysteines, which may undergo *N*-acylation (reaction 8) or an additional transpeptidation reaction to form the corresponding γ-glutamyl derivative (reaction 3a).

Reactive oxygen species and their peroxy products are detoxified by the glutathione redox-cycle. Glutathione acts as a radical scavenger with the redox-active sulphydryl group. In presence of oxygen or reactive oxygen species (ROS) the extracellular or intercellular conversion of GSH to GSSG occurs, which is associated with the formation of H_2O_2 (Figure 1, reaction 12).

Glutathione peroxidase catalyses the reduction of H_2O_2 and other peroxides which is associated with the intracellular conversion of GSH to GSSG (Figure 1, reaction 9). While mammals contain a selenium-dependent glutathione peroxidase, plant glutathione peroxidases do not contain selenium and have more sophisticated roles e.g. removal of lipid and alkyl peroxides (see Foyer and Noctor in this volume).

Transhydrogenation mediates another conversion of GSH to GSSG and the concomitant reduction of disulphides (Figure 1, reaction 10). The reduction of GSSG to GSH is catalysed by the widely distributed enzyme glutathione reductase, which uses NADPH as reductant thereby forming a closed system (redox cycle; Figure 1, reaction 11).

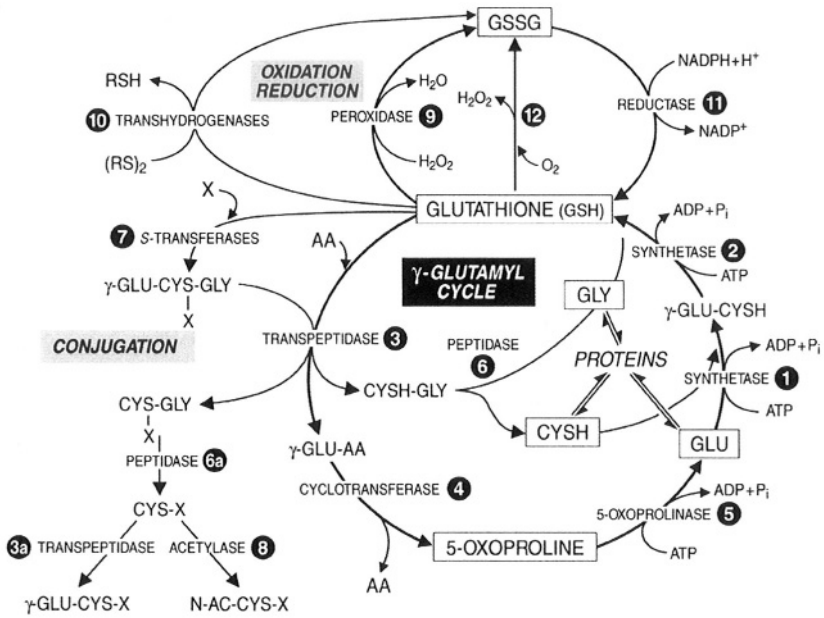


Figure 1. Overview of glutathione metabolism modified from Meister A. and Anderson M. E. (Meister and Anderson 1983).

The BSO model for the investigation of GSH functions

Amino acid sulphoximines are specific inhibitors of γ-glutamylcysteine synthetase, the key enzyme in GSH synthesis. The most commonly employed is buthionine sulphoximine (BSO). BSO decreases the GSH level by 80 to 90% in comparison to control cells. It inhibits the re-synthesis of GSH, which replaces the GSH that is normally exported from the cell.

Deficiency of GSH demonstrates the need for cellular protection from endogenous ROS. It is of particular importance for mitochondria, where the remaining glutathione turns over very slowly. Mitochondria do not synthesize GSH, but import it from the cytosol. Due to the lack of catalase mitochondria are particularly dependent on the protection of GSH and glutathione peroxidase.

GSH monoesters and ascorbate are effective antioxidants, which counteract the damaging effects of ROS. Thus, ascorbate spares GSH and there exist apparently overlapping functions between ascorbate and GSH as well as between GSH and catalase.

A better understanding of basic mechanisms will be able with genetically manipulated yeast strains e.g. glutathione-deficient mutants (*gsh1* and *gsh2*) with stable low GSH levels. These mutants for example are still viable although they have a slower growth rate and show a defect in sporulation. *gsh1* mutants are absolutely dependent upon exogenous GSH for growth in minimal medium and display a petite phenotype, being unable to grow on non-fermentable carbon sources such as glycerol. On the other hand, *gsh2* mutants can grow in unsupplemented minimal media due to the availability of γ-glutamylcysteine (Jamieson 1998).

BIOCHEMICAL REACTION MECHANISMS

The biochemical reactions have been reviewed by Kosower 1989. The most frequent reactions are oxidation-reduction reactions and nucleophilic displacement reactions of the thiol group.

Oxidation-reduction reactions

Both one-electron and two-electron processes of GSH are important in biological and biochemical systems. The sulphur atom of the thiol group is subject to oxidation by one-electron reactions (formation of free thiyl radi-

cals). Furthermore, the thiol group participates as a nucleophile in two-electron reactions.

One-electron reactions

GSH reacts preferentially with free radicals, such as oxygen-, carbon- or nitrogen-centered radicals, whereas reducing (electron-donating) radicals such as pyridinyl radicals are unreactive toward thiols. In these reactions GSH donates hydrogen atoms, which is one of the most important biological function of glutathione. This hydrogen donor activity results in the production of the thiyl ($GS^{\cdot}$) radical. Thiyl radicals are sufficiently stable so that they are readily formed and unreactive toward other hydrogen donors. The formation of the disulphide GSSG results from dimerisation of these radicals:

Hydrogen abstraction → thiyl radical: $RSH + X^{\cdot} \Rightarrow RS^{\cdot} + XH$

$[RS^{\cdot} + RS^{\cdot} \Rightarrow RSSR]$

Other one-electron reactions are the electron abstraction from the thiolate anion by photo-ionisation or by metal ions:

Photo-ionisation: $RS^{-} \Rightarrow RS^{\cdot} + e^{-}$

Electron transfer to metal ion: $RS^{-} + M^{2+} \Rightarrow RS^{\cdot} + M^{+}$

An additional example is the partial reduction of oxidized glutathione:

One-electron reduction to disulphides: $RSSR + e^{+}_{aq} \Rightarrow RSSR^{-}$

$[RSSR^{-} \Rightarrow RS^{-} + RS^{\cdot}]$

The thiyl radical

The bulk of intermediates formed from thiol-reactions are free radicals and sulphenyl derivatives. The (a) donation of a hydrogen atom, or (b) one-electron oxidation of a thiolate anion, or (c) photo-dissociation of certain disulphides leads to the formation of the thiyl free radical. For example the reaction of the hydroxyl radical (OH.) with GSH yields $GS^{\cdot}$:

$$GSH + OH^{\cdot} \Rightarrow GS^{\cdot} + H_2O$$

The thiyl radical forms complexes with anions according to the equation:

$$GS^{-} + GS^{\cdot} \Rightarrow GSSG^{-}$$

and is difficult to detect in any readily accessible spectroscopic region, due to the weak absorption (λ_{max} 330 nm).

The glutathione and L-cysteinyl thiyl radicals react with oxygen at diffusion-controlled rates. The resulting peroxy radical reacts with cysteine or glutathione to yield a sulphenyl hydroperoxide (Cys-SOOH or G-SOOH). The hydroperoxides may react with another thiol to yield a disulphide and hydrogen peroxide:

$$G\text{-}S^{\cdot} + O_2 \Rightarrow G\text{-}SOO^{\cdot} \text{ (peroxy radical)}$$
$$G\text{-}SOO^{\cdot} + Cys\text{-}SH \Rightarrow Cys\text{-}S^{\cdot} + G\text{-}SOOH \text{ (sulphenyl hydroperoxide)}$$
$$G\text{-}SOOH + G\text{-}SH \Rightarrow G\text{-}SS\text{-}G + HOOH$$

Two-electron reactions

The conversion of two thiols into a disulphide can formally be regarded as a two-electron process:

Two-electron oxidation (formal): $RS^- \Rightarrow RS^+ + 2\,e^-$

One-electron oxidation (formal): $RS^- + RS^+ \Rightarrow RSSR$

However, the two-electron oxidation of the thiolate ion, RS^-, to the sulphenium ion, RS^+, is apparently not known, nor is the sulphenium ion a known intermediate.

Known two-electron processes occur e.g. in the displacement reaction of thiols with molecular iodine and in the addition reaction of thiols with double bonds.

Two-electron oxidation by:

1. displacement: $RS^- + I_2 \Rightarrow RSI + I^-$
2. nucleophilic addition: $RS^- + YCO\text{-}N{=}N\text{-}COY \Rightarrow YCO\text{-}N(RS)\text{-}NH\text{-}COY$ (H^+)

The resulting iodide, RSI, of the displacement reaction can further react with a second thiolate anion to give the disulphide in a one-electron oxidation-reduction:

One-electron oxidation-reduction by displacement: $RS^- + RSI \Rightarrow RSSR + I^-$

Thus the formation of GSSG from GSH can occur via radical and non-radical mechanisms.

Nucleophilic reactions

The thiol group of GSH reacts as a nucleophil with electrophiles such as conjugated carbonyls. In these highly reactive electrophiles both the C=C double bond may react according to the equation (Figure 1, reaction 7):

$$GSH + R\text{-}CH{=}CH\text{-}COR \Leftrightarrow R\text{-}CH(SG)\text{-}CH_2\text{-}COR$$

or the carbonyl group as well. These reactions are important detoxification mechanisms of toxic products produced by plants or xenobiotics. Furthermore, they reflect the ability of conjugated carbonyls to inactivate low-molecular weight and protein thiols in biological systems. The equilibrium constants as well as the rate constants for forward and reverse reaction are extremely dependent on the carbonyl structure, e.g. the mother compound acrolein reacts more rapidly than any other carbonyl to give very stable adducts (half-lives for reverse reaction 4.6 days). 4-Hydroxy-2-alkenals, which derive from polyunsaturated fatty acids, are somewhat less reactive forming also very stable adducts showing half-lives between 3.4 and 19 days. Thus, the biological activity of aldehydic lipid peroxidation products is primarily determined by the reactivity of conjugated carbonyls towards thiol groups and due to the stability of the adducts (Esterbauer et al. 1975).

In neutral solutions glutathione reacts spontaneously with 4-hydroxyalkenals, e.g. 4-hydroxy-nonenal, to a saturated aldehyde with the glutathione residue bound by a thio-ether linkage at carbon atom 3, but the principal end product (95 %) in aqueous solution is a five-membered cyclic hemi-acetal, which results from an intramolecular rearrangement of the initial product (Esterbauer et al. 1991).

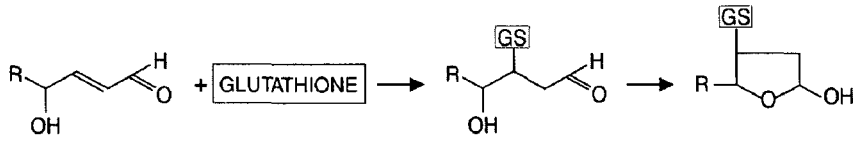


Figure 2. Reaction of glutathione with 4-hydroxyalkenals modified according to Esterbauer et al. 1991.

Plant products as inducers of glutathione *S*-transferases

Glutathione *S*-transferases, which occur in plants, several vertebrate species, invertebrates, and microorganisms, are an important group of detoxification enzymes, which catalyse conjugation reactions between GSH and a

variety of electrophilic compounds, including many environmental toxins (Lau et al. 1980).

Among the toxins detoxified by glutathione *S*-transferases are several carcinogens. Most chemical carcinogens require activation to reactive electrophilic forms by phase I enzymes (cytochromes P-450) in order to exert their toxic and neoplastic effects. The resultant electrophiles are susceptible to metabolic conjugation and other types of detoxifications by phase II enzymes, which include glutathione transferases, NAD(P)H:quinone reductase, and UDP-glucuronosyl-transferases. The balance between phaseI and phaseII enzymes is an important determinant of whether exposure to carcinogens will result in toxicity and neoplasia (Talalay et al. 1990).

Mammalian cells have evolved elaborate mechanisms for protection against the toxic and neoplastic effects of electrophilic metabolites of carcinogens and reactive oxygen species. Glutathione transferases and high intracellular levels of GSH play an important role in providing such protection. Glutathione *S*-transferases are grouped into four classes, α, μ, π, and θ. Some of these forms act to prevent carcinogenesis by detoxifying carcinogens. GST M1 and T1 loss is associated with a greater susceptibility to some cancers.

The regulation of phase II enzymes seems to be mediated by the same enhancer element that contains AP-1-like sites (Prestera et al. 1993). They are transcriptionally- induced by low concentrations of a great variety of chemical agents. Some inducers are widely distributed in edible plants. In broccoli an isothiocyanate sulphoraphane is a very potent inducer of phase II enzymes and blocks mammary tumour formation in rats (Talalay et al. 1995).

Alkylation reactions

GSH reacts with alkyl halides via nucleophilic displacement to form sulphides:

$$\text{G-SH} + \text{R}'\text{X} \Rightarrow \text{G-SR}' + \text{HX}$$

If a second, sufficiently reactive halide molecule is reacted with the sulphide, a sulphonium ion can be formed:

$$\text{G-SR}' + \text{R}''\text{X} \Rightarrow \text{G-S}^{+}\text{R}'\text{R}''$$

Sulphonium ions are reactive toward nucleophiles such as GS^{-}, normally undergoing displacement of a sulphide to yield an alkylated nucleophile:

$$GS^- + G\text{-}S^+R'R'' \Rightarrow G\text{-}S\text{-}G + R'\text{-}S\text{-}R''$$

The most extensively investigated alkylation reaction with glutathione involves halobimanes, because they form the basis for procedures of GSH analysis. Bromoalkyl bimanes are fairly reactive alkyl halides. For example, monobromobimane is 13 times as reactive as benzyl bromide and 43 times as reactive as chlorobimanes toward glutathione. Glutathione derivatives of syn-bimanes are strongly fluorescent and thus applied for glutathione detection accomplished by reverse-phase HPLC analysis (Fahey and Newton 1987, Newton et al. 1981, Anderson 1985).

Thiol-disulphide interchange reactions

The thiol-disulphide interchange reaction is important in maintaining the thiol status of cells (Figure 1, reaction 10):

$$RS^- + R'SSR' \Rightarrow RSSR + R'S^-$$

The activation parameters for the thiolate-disulphide exchange reactions resemble those for other nucleophilic displacement reactions of thiols, suggesting that the exchange is a nucleophilic displacement on sulphur.

The thiol status of a biological system may be described as the distribution of thiols, exogenous or endogenous, among different chemical forms, including thiols (GSH, protein thiols), thiol esters (acyl-CoA) and disulphides (GSSG, CoASSCoA, protein-GS mixed disulphides, and protein-protein disulphides).

A quantitative expression of thiol status can be given as follows:

Thiol status (TS): thiol, directly measurable forms, mM / total, all forms of thiols and disulphides, mM. (The total includes all types of SH and SS equivalents.) The experimental methods for both quantities must be specified. Thus, for human red blood cells: TS = 3.5 / 4.0.

The relationship of thiol status to enzyme function, metabolic regulation, and cellular structure is not well understood. An enzyme that may be important in regulating thiol status is thiol-transferase.

The redox status of GSH (the ratio GSH/GSSG) is closely related to the thiol status. It is an important parameter for the antioxidant defence and is always markedly shifted in favour of the reduced form. Thus, in spinach chloroplasts, where the activity of glutathione reductase is high while glutathione peroxidase is absent, the ratio GSH/GSSG is >10/1, and in *Neurospora crassa* (moderate glutathione reductase level; glutathione peroxidase absent) the reduced form is even more dominating (150/1).

Concluding remarks

In plant cells GSH fulfils several functions. As an antioxidant GSH protects cell constituents against oxidative stress, e.g. GSH - in cooperation with ascorbate - removes reactive oxygen species which are formed as by-products of the photosynthesis. Furthermore, GSH accomplishes protective functions by conjugation of xenobiotics and as starting material for the synthesis of phytochelatins for the detoxification of heavy metals. Additionally, GSH serves as storage compound for organic sulphur. Through enzymatic degradation cysteine can be recovered if needed. Novel functions of GSH are emerging (Sies 1999). One is the hopping of nitric oxide between thiol groups in a process known as trans-nitrosation (Al-Mustafa et al. 2001). A major focus of current research is directed to the role of thiols in signalling, i.e. the control and modulation of pathways leading to gene expression (Sies 2001).

Currently, the prospects for the molecular enhancement of glutathione are being explored (Foyer 2001, Anderson 1997). GSH biosynthesis has been studied extensively in C_3 plants by overexpressing γ–glutamyl cysteine synthetase and glutathione synthetase, and a substantial constitutive increase in tissue GSH has thereby been achieved. However, such molecular genetic approaches are not acceptable to the consumer and alternative approaches have to be found. The possibilities for using the plants' own defences against oxidative stress to improve the antioxidant content of plant foods will be explored using GSH and ascorbate as models. The signals involved in triggering high antioxidant accumulation are only poorly understood. Leaves have a high capacity for production of one well-known signal, H_2O_2. Intracellular H_2O_2 concentrations are low, however, because of control by the antioxidant system. Mutants and transformed plants with specific decreases in key components offer the opportunity to dissect the complex system that maintains redox homeostasis and determines the antioxidant responses.

Further information of importance may be found in several books and reviews, e.g. references (Meister and Anderson 1983, Lamoureux and Rusness 1989, Meister 1983, Sies 1999, Anderson 1997, Wonisch et al. 1997, Heldt and Heldt 1996).

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Chapter 3

THE MOLECULAR BIOLOGY AND METABOLISM OF GLUTATHIONE

Christine H. Foyer and Graham Noctor

INTRODUCTION

Glutathione (γ-glu-cys-gly) is a major reservoir of non-protein reduced sulphur in plants, animals and other organisms. Some plants contain other thiol tripeptide homologues of glutathione, in place of or in addition to glutathione, where another amino acid replaces the carboxy-terminal glycine. Homoglutathione (hGSH; γ-glu-cys-β-ala) is found in the Fabaceae (Price 1957, Klapheck 1988) and Poaceae contain γ-glu-cys-ser (Klapheck et al. 1992) while, under certain conditions, γ-glu-cys-glu is found in maize (Meuwly et al. 1993). Diurnal changes in the amounts of glutathione are observed in leaves (Noctor et al. 1998b). Developmental, environmental and seasonal factors all cause variations in tissue glutathione contents (and, where they are present, in those of its homologues). Glutathione biosynthesis is increased in response to biotic threats and environmental stresses. Such observations led May et al. (1998a) to suggest that glutathione could act as a direct link between environmental stress and a number of key processes such as mitosis and root development in plants. The following analysis embraces this idea and emphasizes the integration of glutathione biosynthesis into cellular physiology, as well as the universal roles of glutathione in signal transduction and in plant defence responses to stresses that involve production of active oxygen species (Figure 1).

THE FUNCTIONS OF GLUTATHIONE: MOLECULAR AND BIOCHEMICAL ASPECTS

In addition to the storage and transport of reduced sulphur, glutathione and/or its homologues have a broad spectrum of functions in plants and animals (Figure 2). These include (A) antioxidant defence, (B) redox regulation

D. Grill et al. (eds.), Significance of Glutathione to Plant Adaptation to the Environment, 27-56.

and buffering, (C) regulation of gene transcription and translation, (D) modulation of enzyme activity, (E) modification and transport of hormones and other endogenous compounds, as well as xenobiotics, via formation of glutathione *S*-conjugates.

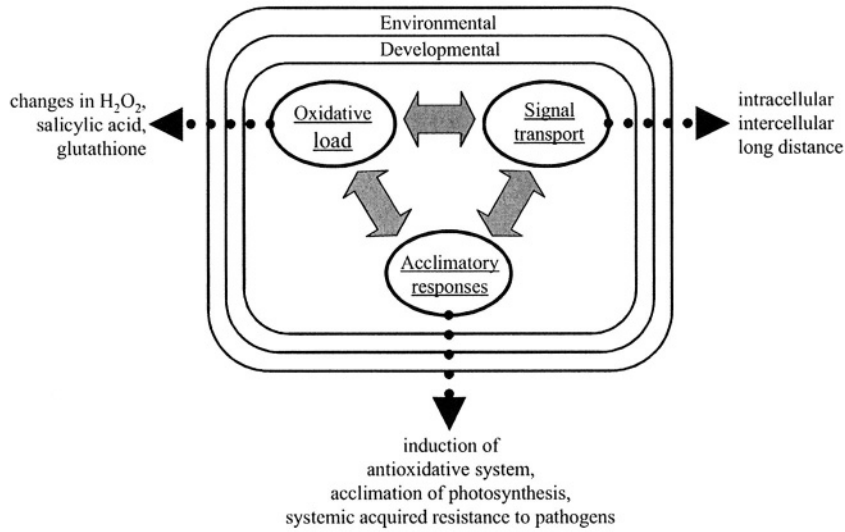

Figure 1. Signal-response relationships in photosynthetic metabolism and plant-pathogen interactions: the possible role of glutathione.

While it has long been accepted that glutathione is essential for vigour, it has only recently been recognized that this tripeptide cannot be functionally replaced, except perhaps by one of its homologues. The *rml1* mutant of *Arabidopsis,* which is deficient in γ-ECS and contains no detectable glutathione, has a marked phenotype with an absence of root development and a small shoot system, and can survive only in tissue culture supplied with GSH (May et al. 1998a). A strong correlation has also been demonstrated between root GSH content and the capacity of the cells in the root apical meristem to proliferate (Sanchez-Fernandez et al. 1997).

Glutathione is a general protector of cell function. It reacts chemically with a range of active oxygen species (AOS), while enzyme-catalysed reactions link GSH to the detoxification of H_2O_2 via the action of glutathione reductase (GR) in the ascorbate-glutathione cycle, and to the removal of organic peroxides via glutathione peroxidase (GPX). GSH protects proteins against the denaturation that is caused by oxidation of protein thiol groups during stress.

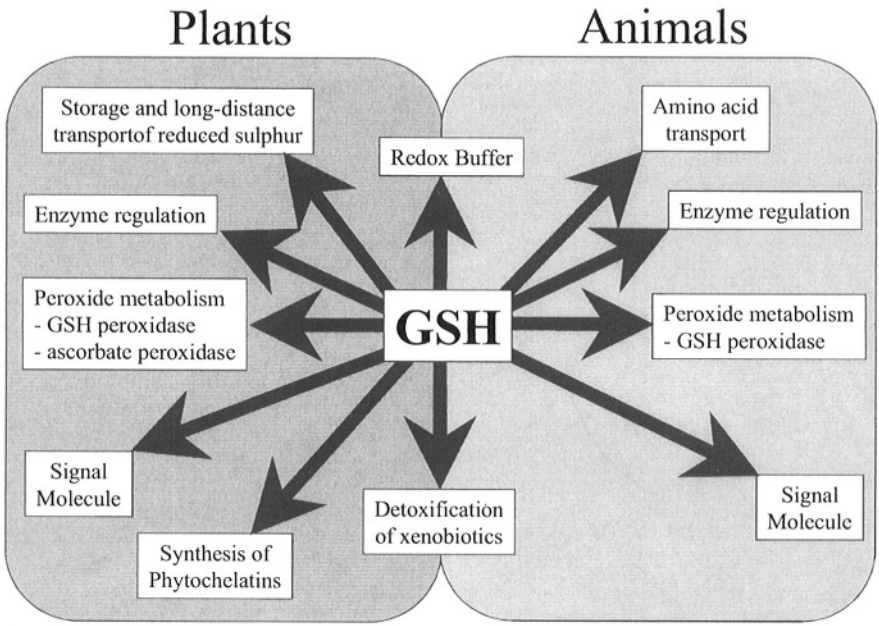

Figure 2. Functions of glutathione in animals and plants.

H_2O_2-mediated orchestration of gene expression may be central to the ability of plants to elicit antioxidative defences in response to abiotic and biotic threats and hence develop pre-emptive cross-tolerance. Similarities between the oxidative stress caused by ozone fumigation and pathogen-induced responses suggest common elements in signal transduction routes involving salicylic acid, jasmonic acid (JA) and ethylene (Rao and Davis 1999). Transformed tobacco plants, deficient in the H_2O_2-scavenging enzyme catalase, also show symptoms that are linked to the activation of pathways involved in apoptosis, such as the induction of pathogenesis-related (PR) proteins (Chamnongpol et al. 1996, 1998). Hydrogen peroxide-mediated induction of glutathione has been demonstrated in different systems (May and Leaver 1993, May et al. 1996). As discussed below, glutathione also accumulates when catalase activities are low.

AOS modulate nitric oxide signalling in HR, leading to apoptosis in cells attacked by pathogens (Delledonne et al. 1998) and systemic acquired resistance (SAR) in surrounding tissues. SAR involves the pre-emptive deployment of gene expression to modify cell metabolism to cope with future attacks. Increases in the phenolic signal molecule salicylic acid (SA) are ob-

served at the site of infection, and to a lesser extent at remote sites. H_2O_2 and SA (and perhaps also glutathione) are potential systemic messengers carrying information concerning attack to unchallenged plant tissues (Figure 1). Of the complex array of antioxidants found in plant cells, to date glutathione alone has been shown to be strongly induced and to accumulate rapidly in response to pathogen attack (Edwards et al. 1991, Vanacker et al. 2000). The effect on biosynthesis is specific to GSH and not a general effect on the synthesis of low molecular weight antioxidants. It appears to be a universal response in plants faced by pathogen attack or environmental stress, where the antioxidant defences are temporarily overwhelmed by an oxidative burst or by the accumulation of AOS as a result of impaired metabolism.

The signalling mechanisms involved in induction of GSH biosynthesis during pathogen attack are unknown. It is clear that whereas H_2O_2 increases tissue GSH contents, JA increases the transcript abundance of the enzymes of GSH synthesis but does not affect GSH concentration (Xiang and Oliver 1998). Pathogen-induced increases in glutathione in the cells surrounding the site of attack may have two possible roles in defence. First, they would increase protection from excessive damage caused by the accumulation of active oxygen species (AOS) during the oxidative burst (May et al. 1996). This may occur chemically or through increased substrate availability for enzymes such as GSTs and GPXs. Second, changes in the redox state and concentration of glutathione may be an essential secondary messenger mediating the signalling effects of hydrogen peroxide (Foyer et al. 1997, May et al. 1998a).

Intrinsic to signalling process involving glutathione are changes in the GSH:GSSG ratio, because these lead to changes in gene expression. GSH and GSSG may themselves potentiate the signal but a second putative mechanism of action has recently been highlighted. This involves the spontaneous oxidation of protein sulphydryl groups to form disulphides with low molecular weight thiols (such as GSH), a reaction termed thiolation. Thiolation may be a crucial initial signalling event that initiates responses to oxidative stress and pathogens. The formation of such intramolecular disulphide bonds within proteins alters their configuration and biological activity (Demple 1998). Reversible protein thiolation protects essential thiol groups on key proteins from irreversible inactivation during oxidative stress (Thomas et al. 1995) and also plays an important regulatory role in controlling metabolism, protein turnover and gene transcription (Jahngen-Hodge et al. 1997). There are many examples of proteins that undergo thiolation in animals but relatively few have been described in plants. Spinach seed acyl carrier protein is thiolated in the latter stages of seed development and dethiolated during imbibition (Butt and Ohlrogge 1991). In this case, thiolation is linked to inactivation; in other cases, activation results. Thiolation has been found to activate microsomal GSTs (Dafré et al. 1996) and nuclear

factor I transcription factors from HeLa cells (Bandyopadhyay et al. 1998). In this way, thiolation in plants may represent a powerful mechanism for direct post-translational regulation of metabolism. Thiolation of proteins such as phosphotyrosine-specific protein phosphatases may also mediate signal transduction pathways that initiate key stress responses (Fordham-Skelton et al. 1999).

Regulation of gene expression by GSH and GSSG may be specific, i.e., these compounds may be irreplaceable by other redox components. Alternatively, reported effects may reflect general changes, in cellular redox state. Application of exogenous glutathione can elicit changes in the transcription of genes encoding cytosolic Cu,Zn SOD and GR in tobacco and pine (Hérouart et al. 1993, Wingsle and Karpinski 1996) and 2-cys peroxiredoxins in *Arabidopsis* (Baier and Dietz 1997). Glutathione-inducible hypersensitive elements have been identified in the proximal region of the CHS promoter in *Phaseolus vulgaris, Medicago sativa* and *Glycine max.* (Dron et al. 1988, Choudhary et al. 1990, Lawton et al. 1990). Although pathogen-induced increases in the intracellular concentration of glutathione and GSH-dependent induction of PAL and CHS have been demonstrated (Wingate et al. 1988), it is unlikely that GSH is the primary signal responsible for the increase in phytoalexins following pathogen attack. Using an artificial precursor of glutathione biosynthesis, L-oxothiazolidine-4-carboxylate, to increase intracellular thiol concentrations, Edwards et al. (1991) showed that enhanced intracellular GSH concentrations did not induce phytoalexin synthesis. They concluded that changes in the intracellular glutathione concentration in response to pathogen attack were too slow to be consistent with the initiation of the elicitation response. In interactions between powdery mildew and oat or barley, however, induction of glutathione precedes maximal induction of transcripts for phenylpropanoid metabolism (Zhang et al. 1997a, Vanacker et al. 2000). There are differences in the signal transduction pathways for elicitation of CHS transcription by fungal elicitor and glutathione (Choudhary et al. 1990), suggesting that increases in glutathione are not primarily responsible for elicitation of the defence response. A role for glutathione in subsequent parts of the signal transduction pathway is nevertheless possible.

In prokaryotes and eukaryotes, gene expression can be regulated by cellular redox status. In *Arabidopsis* and tobacco the promoters of individual GST genes containing a consensus sequence termed the *ocs* (or *as-1*-type) element, have been shown to be involved in the response not only to auxin and salicylic acid but also to changes in redox state (Chen et al. 1996, Xiang et al. 1996). The glutathione pool is an important redox component in plant cells (Kunert and Foyer 1993). Changes in the intracellular glutathione concentration may therefore be expected to have important consequences for the

cell with regard to the metabolic functions associated with the products of genes that can be regulated by glutathione. In animal cells redox regulation of the transcription factor NFκB involves glutathione. This regulation is important for T cell function since glutathione augments the activity of T cell lymphocytes (Suthanthiran et al. 1990).

GLUTATHIONE PEROXIDASE, GLUTATHIONE *S*-TRANSFERASE, GLUTATHIONE REDUCTASE

The selenoprotein glutathione peroxidase (GPX) detoxifies H_2O_2 in animal tissues but an equivalent reaction is not found in plants. Plant GPXs are not constitutive but are induced in response to stress. They do not contain selenium and do not catalyse the GSH-dependent reduction of H_2O_2 (Eshdat et al. 1997). In plants ascorbate peroxidase (APX) and catalase (CAT) detoxify H_2O_2 while GPXs have more sophisticated roles, including the removal of lipid and alkyl peroxides (Kranner and Grill 1996, Eshdat et al. 1997). Kranner and Grill (1996) suggested that GPXs may be involved in the GSH-dependent regeneration of α-tocopherol. In addition, GSTs can function to remove lipid peroxides and fulfil a peroxidatic role (Bartling et al. 1993, Cummins et al. 1999). Oxidative stress induces specific GST isoforms and GPXs whose role is to detoxify lipid peroxidation products. Transgenic tobacco lines overexpressing plant GST/GPX were reported to show enhanced antioxidant capacity and substantial improvement in seed germination and seedling growth under stress (Roxas et al. 1997). While GPXs may reside in many organelles, the only clear demonstration of GPX targeting thus far has shown direction to the chloroplast (Mullineaux et al. 1998). Most studies involving GSTs have focused on their role as cytosolic enzymes, but they may also be found in other compartments. In animals, GST isoforms are found in microsomes (Dafré et al. 1996) and activity may be associated with leaf peroxisomes in plants.

GSTs form a large, heterogeneous family of proteins that share the defining characteristic of catalysing the nucleophilic attack of the sulphur atom of glutathione (or analogue) on the electrophilic centre of their substrates (Lamoureux and Rusness 1993, Mannervik and Danielson 1998). They are therefore responsible for the removal of compounds that are potentially genotoxic or cytotoxic by virtue of their reaction with nucleophilic sites in DNA, RNA and proteins. It has become clear, however, that the function of GSTs is not limited to these reactions: GSTs are also involved in a 'ligandin' function important, for example, in the anthocyanin synthesis pathway. This enables transfer of the precursor to the vacuole (Marrs et al. 1995, Alfenito et al. 1998). The exact mechanism of these reactions is not clear, being

somewhat obscured by the fact that the substrates are poor electrophiles. By virtue of the defensive action of GSTs, xenobiotics (e.g., fluorodifen, atrazine, metolachlor) are detoxified, and endogenous secondary compounds and other products (auxins, anthocyanins, organic peroxides, quinones, sulphate esters, cinnamic acid, medicarpin) are sequestered in the vacuole. At least some conjugates appear to be tagged for transport from the cytosol to the vacuole by a Mg-ATP glutathione *S*-conjugate transporter, which is up-regulated along with GSTs upon exposure to xenobiotics (Martinoia et al. 1993, Li et al. 1995). The glutathione *S*-conjugates formed with anthocyanin and medicarpin are also transported into the vacuole by a specific glutathione *S*-conjugate transporter (Marrs et al. 1995, Li et al. 1997, Alfenito et al. 1998), where they are further metabolized (Marrs 1996).

Cellular GSH:GSSG ratios are maintained by glutathione reductase (GR), a homodimeric flavoprotein that uses NADPH to reduce GSSG to two GSH. In leaves, the bulk of GR activity is found in the chloroplast whereas root plastids may contain a lower proportion of the total cellular activity (Foyer and Halliwell 1976, Bielawski and Joy 1986, Edwards et al. 1990). About 20 % of the pea leaf activity was associated with the cytosol (Edwards et al. 1990). Lower GR activities have been reported in isolated mitochondria and peroxisomes (Edwards et al. 1990, Jimenez et al. 1997). The first gene sequences encoding plant GR were isolated from pea and *Arabidopsis*, and shown to encode products with N-terminal sequences characteristic of chloroplast-targeting sequences (Creissen et al. 1992, Kubo et al. 1993). Interestingly, expression of the pea gene in tobacco showed that the product was targeted to both chloroplasts and mitochondria (Creissen et al. 1995). Subsequently, other cDNAs have been isolated from tobacco, pea, rice and *Brassica campestris*, some of which encode cytosolic isoforms (Creissen and Mullineaux 1995, Stevens et al. 1997, Kaminaka et al. 1998, Lee et al. 1998). Analysis of whole leaf GR preparations from pea and spinach by isolectric focusing revealed multiple bands (Edwards et al. 1990, Foyer et al. 1991). Since no evidence was found for post-translational modifications, mutiple proteins may reflect expression of a gene family or differences in RNA splicing during transcript processing (Creissen and Mullineaux 1995).

In the light, chloroplastic GR activity is linked to the thylakoid electron transport chain via NADPH oxidation. While the enzyme can use NADH, it has a much lower affinity for this nucleotide (Edwards et al. 1990, Kubo et al. 1993). Mitochondrial GR was found to show similar low affinity for NADH (Edwards et al. 1990) or to display an absolute requirement for NADPH, which can be produced in the matrix by some TCA cycle enzymes (Rasmusson and Møller 1990). In maize, the almost exclusive localization of GR activity in the mesophyll cells may be explained by the comparative lack of reductant in the bundle sheath cells, which have very low amounts of

Photosystem II (Doulis et al. 1997). The absence of GR from the maize bundle sheath is due to post-transcriptional regulation, since GR transcripts are found in both cell types (Pastori et al. 2000).

Overexpression of either plant or bacterial GR can increase leaf GSH:GSSG ratio and mitigate damage due to certain stresses (Foyer et al. 1991, 1995, Aono et al. 1993, Broadbent et al. 1995). Interestingly, several studies have shown a correlation between GR activity and the absolute size of the glutathione pool. Increases in GR activities or expression are induced by various treatments, including H_2O_2, ozone, paraquat, chilling, drought, and abscisic acid (Pastori and Trippi 1992, Kaminaka et al. 1998, Lee et al. 1998). Induction of GR activities appears to reflect control at the transcriptional and/or translational level: there is as yet little evidence for regulation of plant GR activities by post-translational modification or by metabolite effectors.

BIOSYNTHESIS: REGULATION OF GENE EXPRESSION AND ENZYME ACTIVITIES

The pathway of glutathione biosynthesis is well established and is similar in plants, animals and micro-organisms. In two ATP-dependent steps, catalysed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), the constituent amino acids are linked to form the complete tripeptide. The N-terminal peptide bond linking glutamic acid to cysteine in GSH is unusual in that glutamic acid is linked via the γ– rather than the α– carboxyl group. The two-step reaction sequence occurs in both chloroplastic and non-chloroplastic compartments and is found in photosynthetic and non-photosynthetic tissues (Law and Halliwell 1986, Klapheck et al. 1987, Hell and Bergmann 1988, 1990, Ruegsegger and Brunold 1993, Noctor et al. 1998a, Noctor and Foyer 1998). The existence of a mitochondrial isoform of γ-ECS in *Brassica juncea* has been postulated (Schäfer et al. 1998).

In maize, which has strong demarcation of metabolism within photosynthetic cells, including antioxidants (Doulis et al. 1997), cysteine is synthesized in the bundle sheath whereas GS activity is located predominantly in the mesophyll cells (Burgener et al. 1998). It appears, therefore, that glutathione is synthesized in the cells where GR is present and that the bundle sheath relies on the mesophyll for the synthesis of glutathione and the reduction of GSSG. At the subcellular level, the distribution of enzyme activity between chloroplast and extra-chloroplastic compartments is approximately equal. Chloroplasts from *Pisum sativum* contained 72 % of leaf γ-ECS activity and 48 % of leaf GS activity (Klapheck et al. 1987, Hell and Bergmann 1990). In *Spinacia oleracea,* values of 61 % (γ-ECS) and 48 % (GS) were

found (Hell and Bergmann 1990). The very low activities of these enzymes in plants and the complexities of the procedures for enzyme extraction and assay have precluded extensive purification and kinetic characterization. Consequently, much of our current knowledge of their structure, regulation and function has been gleaned from molecular techniques and plant transformation, and to date this remains limited.

The gene encoding γ-ECS here denoted as *gsh1,* was originally cloned from *Arabidopsis thaliana* by complementation of an *E. coli* mutant deficient in this enzyme (May and Leaver 1994). Heterologous expression of the *Arabidopsis* γ-ECS in a yeast mutant only recovered 10 % of the GSH measured in the wild-type yeast (May and Leaver 1994). This discrepancy provoked much speculation concerning the identity of the cloned gene, but further complementation studies have now confirmed that this gene does indeed encode a protein with true γ-ECS activity (May et al. 1998a).

Functional complementation of an *E. coli* mutant deficient in GS activity was also used to clone the *Arabidopsis thaliana* gene for this enzyme, which we denote here as *gsh2* (Rawlins et al. 1995, Ullman et al. 1996) The ability of several plant species to make analogues of glutathione depends on the specificity of the synthetases involved. Specific legume GSs use either glycine to form GSH or β-alanine to form homoglutathione (MacNicol 1987). Recent evidence from *Medicago truncatula* suggests that separate genes encode GS and homoglutathione synthetase (hGS) and that the divergence in specificity has arisen by gene duplication after the evolutionary divergence of the *Fabaceaea* (Frendo et al. 1999). The two genes are very homologous and are found on the same fragment of genomic DNA (Mathieu 1999).

Relatively little is known about the co-ordinate regulation of expression of *gsh1* and *gsh2* but it is clear that GSH and GSSG *per se* have no control over the expression of these genes (Xiang and Oliver 1998). Similarly, H_2O_2 did not affect expression. The abundance of *gsh1* and *gsh2* transcripts was increased by cadmium in *Brassica juncea* (Schäfer et al. 1998). Xiang and Oliver (1998) found that both cadmium and copper increased transcript abundance in this species (Figure 3). Interestingly, JA also increased *gsh1* and *gsh2* transcripts and a common signal transduction pathway may be involved (Xiang and Oliver 1998). Most importantly, oxidative stress was required for the translation of the transcripts in these conditions, suggesting that post-transcriptional regulation was involved in the control of GSH synthesis and that H_2O_2 (or low GSH/GSSG ratios) de-represses translation of the existing mRNA (Xiang and Oliver 1998). Studies in animals, particularly on cancer cells challenged with chemotherapeutic agents, have shown that transcription of the γ-ECS gene is regulated by protein factors and by conserved antioxidant response elements upstream of the coding sequence (Figure 3).

	Animals	Plants
Correlative studies:	γ-ECS activities and GSH levels correlate with resistance of tumour cells to chemotherapeutic agents (Godwin et al. 1992, Mulcahy et al. 1994).	γ-ECS activities and GSH levels correlate with resistance or response to Cd exposure (Rüegsegger and Brunold 1992, Chen and Goldsbrough 1994).
Overexpression studies:	Transfection of γ-ECS subunits into human cell lines increases GSH levels about 2-fold (Kurokawa et al. 1995, Mulcahy et al. 1995).	Transformation of poplar to overexpress γ-ECS increases foliar GSH levels about 3-fold (Arisi et al. 1997, Noctor et al. 1998a).
Control and induction:	Oxidative stress induces γ-ECS and elevates GSH content in rat lung epithelial cells (Shi et al. 1994). Involvement of regulatory protein transcription factors and 'antioxidant response elements' upstream of the heavy subunit coding sequence (Rahman et al. 1996, Tu and Anders 1998, Wild et al. 1998, Lapperre et al. 1999).	Exposure to heavy metals or jasmonic acid induces increased transcript levels for γ-ECS and glutathione synthetase (Xiang and Oliver 1998). Does γ-ECS in plants have a second 'regulatory' subunit, as yet uncharacterised, which would be analogous to the light subunit of the mamalian γ-ECS? (May et al. 1998).

Figure 3. Control of GSH synthesis: some recent developments and likely perspectives.

Post-translational regulation of γ-ECS may also be influential. There is some evidence to suggest that rat γ-ECS may be regulated by protein phosphorylation (Sun et al. 1996) but this has not yet been found in studies on plant γ-ECS. May et al. (1998b) concluded that protein factors are involved in post-translational control of γ-ECS and are required for full activity. The failure of the plant enzyme to operate ectopically was explained by the absence of such endogenous plant factors (May et al. 1998a,b). In the animal enzyme system a smaller regulatory subunit acts to increase the catalytic potential of the larger catalytic subunit by increasing its Ki value for GSH and decreasing the Km for glutamate, thereby alleviating feedback control and allowing the enzyme to operate under *in vivo* conditions (Huang et al. 1993). It is nevertheless clear that, in the case of the animal enzyme, the large catalytic subunit is capable of some catalysis, since overexpression of this polypeptide alone yielded increased GSH levels in transfected cells (Kurokawa et al. 1995, Mulcahy et al. 1995). Highest GSH levels were, however, obtained by dual over-expression of both subunits (Mulcahy et al. 1995). While protein factors have not been identified in plants, and there is as yet no evidence for control of γ-ECS by phosphorylation, several cytosolic enzymes in plants are controlled by interactions between phosphorylation status and factors such as 14-3-3 proteins (Moorhead et al. 1999 and references therein). This interaction inactivates enzymes such as nitrate reductase but confers stability against proteolytic attack. It is as yet unclear whether this type of regulation might be important in glutathione synthesis in plants,

but it is perhaps worth considering the possibility that post-translational control of glutathione synthesis occurs through distinct mechanisms in the chloroplast and cytosol.

Whatever the control mechanisms, several studies have demonstrated that, under most conditions, the activity of γ-ECS limits the rate of glutathione synthesis in both plants and animals (Figure 3). This enzyme is responsible for maintaining the GSH concentration of plant cells as demonstrated by the following observations: 1) The *cad-2 Arabidopsis* mutant, which has a mutation in the *gsh1* gene, has only one-third of the tissue GSH contents of the wild-type (Cobbett et al. 1998); 2) increases in glutathione contents accompany increases in γ-ECS in tissues treated with cadmium (Rüegsegger and Brunold 1992). 3) Overexpression of an *E. coli* γ-ECS but not GS, in poplar or tobacco substantially increases the glutathione pool (Noctor and Foyer 1998). 4) Transformed plants expressing the *Arabidopsis gsh1* gene, in either the sense or antisense orientation, have been produced by the group of David Oliver. In these plants the GSH contents ranges from 2 % to 150 % of the untransformed controls. It should be noted, however, that other *Arabidopsis* plants, in which homologous overexpression of the *gsh1* gene led to a decrease in steady-state transcript levels, had similar glutathione contents to control plants (Rawlins 1998).

Bacterial genes encoding γ-ECS and GS have been introduced into poplar, mustard and tobacco (Strohm et al. 1995, Foyer et al. 1995, Noctor et al. 1996, 1998a, Arisi et al. 1997, Zhu et al. 1999, Pilon-Smits et al. 1999, Creissen et al. 1999). Over-expression, with targeting of the bacterial enzyme protein to either the chloroplast or cytosol, led to strong increases in enzyme activity. Increases in γ-ECS but not GS led to constitutive increases in foliar glutathione (Figure 4) of up to 400 % (Noctor et al. 1996, 1998a, Arisi et al. 1997, Creissen et al. 1999). The bacterial enzymes are clearly able to function *in planta* to increase tissue γ-EC pools substantially. Interestingly, the cysteine pool was not depleted by the increased demand for thiols but was even slightly enhanced in response to increased γ-ECS activities, pointing to co-ordinate regulation of cysteine synthesis and glutathione synthesis (Noctor et al. 1998b). Incubation of leaf discs with cysteine increased glutathione contents substantially in untransformed poplars, particularly in the light (Figure 5). Although cysteine treatment of leaf discs from poplars overexpressing γ-ECS also increased glutathione contents, the effect was often less marked in these plants (e.g. data of Figure 5). This difference may be because, in the absence of exogenous cysteine, cysteine supply was improved in the transformants and less limiting for glutathione synthesis.

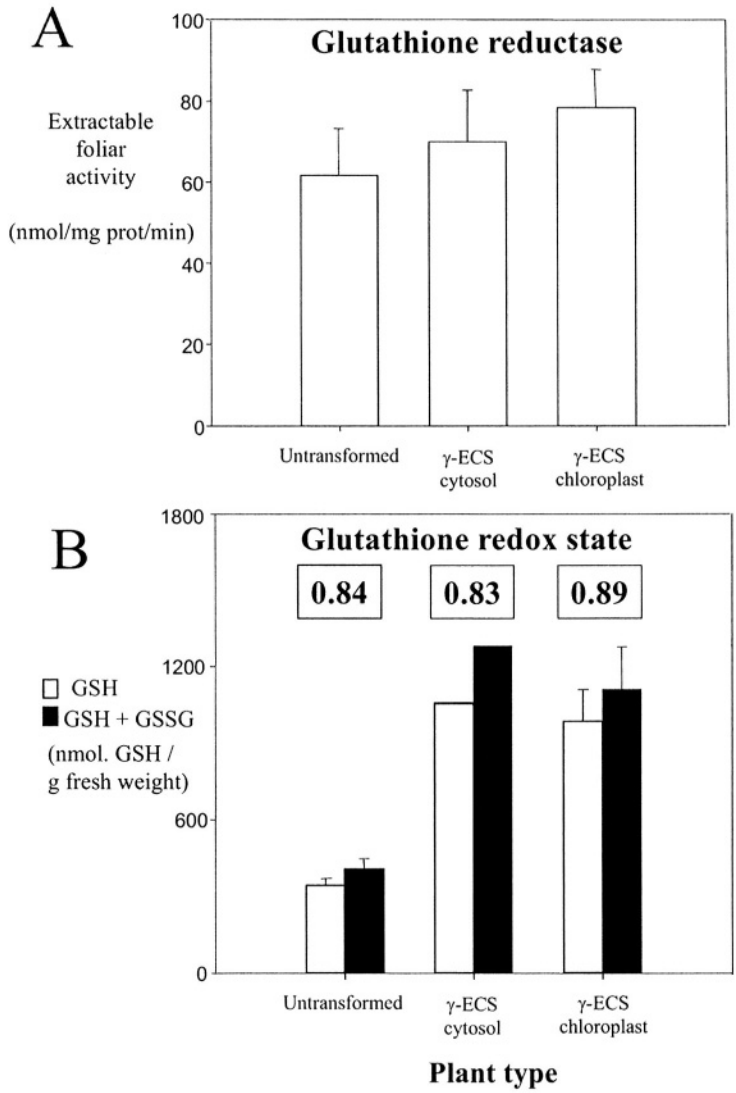

Figure 4. Increased biosynthetic capacity in the chloroplast or cytosol up-regulates glutathione contents without affecting glutathione redox state or extractable GR activity. Poplars were transformed with an *E.coli* γ-ECS gene directed to the cytosol or chloroplast. In both cases, extractable γ-ECS activities were increased to more than 30-fold the foliar activities in untransformed plants. Repeated measurements over a period of 2 years showed that the foliar glutathione pool was increased by an average of 3-fold (B) but that neither extractable GR (A) nor the redox state of the pool (B) were significantly changed. In calculating glutathione redox state, 1 GSSG is taken as equivalent to 2 GSH.

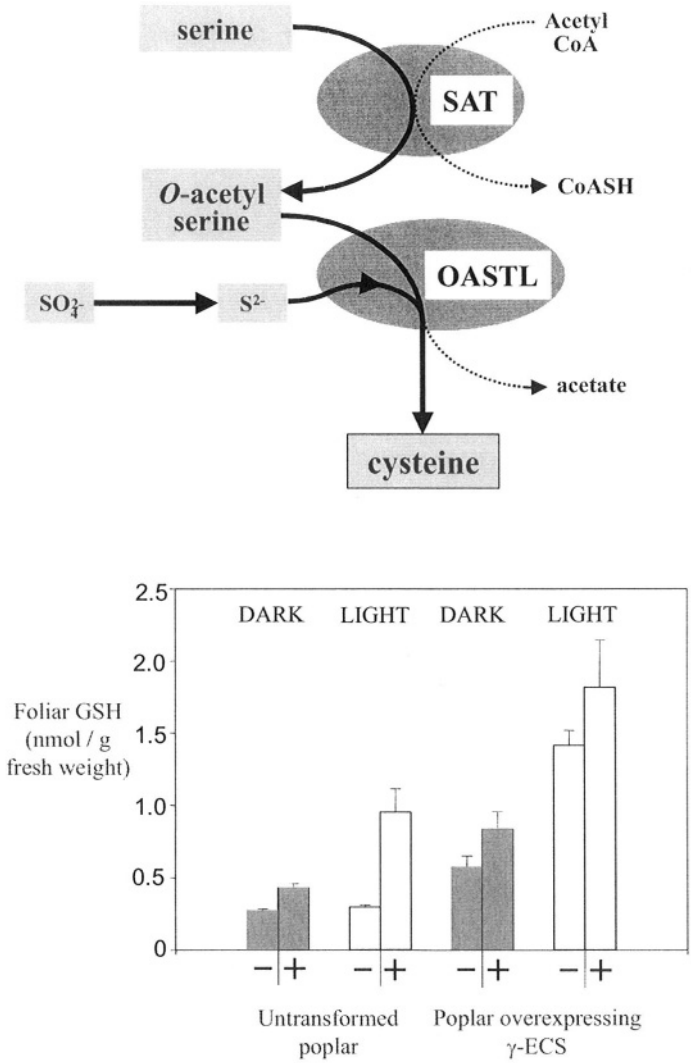

Figure 5. Effect of cysteine and light on glutathione contents in untransformed poplars and poplars overexpressing γ-ECS. The top half of the figure shows cysteine synthesis from serine and sulphide. The bottom half shows the results of an experiment in which leaf discs were incubated in the light or dark in the presence or absence of 5 mM cysteine. SAT, serine acetyltransferase; OASTL, *O*-acetylserine thiol lyase.

The dipeptide produced by the γ-ECS reaction is present at very low levels in most untransformed plants. In the poplars overexpressing γ-ECS, however, γ-EC was greatly increased. The marked increase in γ-EC reflected a shift in control from γ-ECS to GS, whether the bacterial γ-ECS was present in the cytosol or chloroplast (Noctor et al. 1998a). This suggested that overexpression of both enzymes together would increase the potential for constitutive enhancement of tissue glutathione contents even further than that achieved by γ-ECS overexpression alone. This effect was observed when tobacco lines expressing each of the biosynthetic enzymes were crossed to produce hybrids over-producing both enzymes (Creissen et al. 1999). Surprisingly, however Creissen et al. (1999) report that γ-EC contents were higher in the lines overexpressing both enzymes than in those lines overexpressing γ-ECS alone. This anomaly suggests that the effects in tobacco are complex and require caution in interpreting the factors that control glutathione synthesis, particularly since, unlike the poplars overexpressing γ-ECS, the tobacco lines showed marked phenotypes (Creissen et al. 1999). The reasons for the phenotypic differences between tobacco and poplar are not clear but, like the poplars, transformed *Brassica juncea* plants overexpressing the bacterial γ-ECS or GS genes showed normal phenotypes and displayed enhanced tolerance to cadmium (Zhu et al. 1999, Pilon-Smits et al. 1999). Extensive analysis in poplar suggest that the most important factors controlling glutathione contents are the activity of γ-ECS and the availability of cysteine (Figures 4 and 5). It is now apparent that the *in vivo* activity of γ-ECS is controlled at multiple levels. Regulation occurs at the level of transcription and translation, and may also occur post-translationally through protein factors or covalent modification. The susceptibility of the plant enzyme to inhibition by GSH, which is competitive with glutamate (Hell and Bergmann 1990, Schneider and Bergmann 1995), is likely to be an important homeostatic restraint that checks excessive accumulation of glutathione.

INTEGRATION WITH CELLULAR PHYSIOLOGY

Several studies over recent years have highlighted interactions between glutathione metabolism and photorespiration, a process, which in C_3 plants can occur at rates approaching those of net photosynthesis (Foyer and Noctor 2000). The photorespiratory C and N recycling pathways involve at least four amino acids (glutamate, glutamine, glycine and serine) and can produce H_2O_2 at very high rates in the reaction catalysed by glycollate oxidase. Here, we will discuss three types of interaction between photorespiration and glutathione metabolism: 1. utilization of photorespiratory intermediates for glu-

tathione synthesis; 2. a possible role for the ascorbate-glutathione pathway in the processing of photorespiratory H_2O_2; 3. effects on glutathione contents and redox state when catalase activity is decreased (Figure 6).

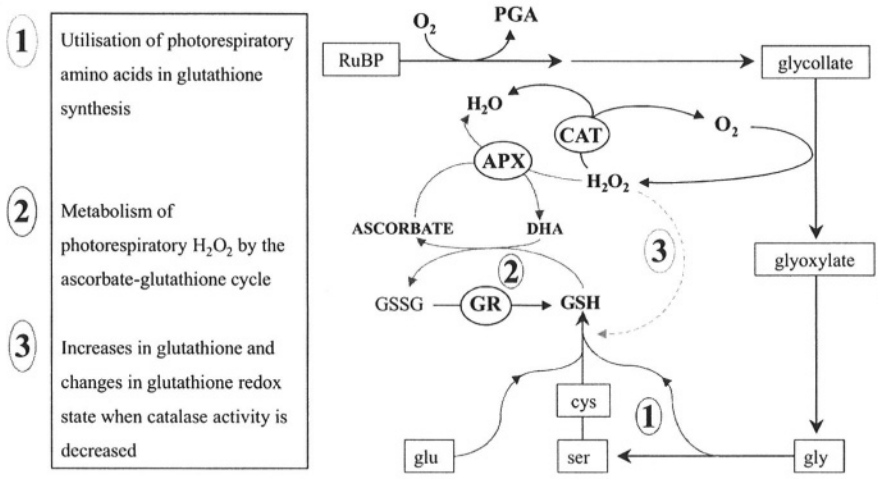

Figure 6. Interactions between glutathione and photorespiration. APX, ascorbate peroxidase, CAT, catalase; DHA, dehydroascorbate; RuBP, ribulose-1,5-*bis*phosphate; PGA, 3-phosphoglyceric acid.

Much attention has focused on interactions between glutathione synthesis and sulphur assimilation, understandably in view of the influence of cysteine concentration on glutathione accumulation (Figure 5). As a tripeptide, however, glutathione is also linked to other pathways of amino acid synthesis. The first evidence that photorespiratory glycine is used in glutathione synthesis came from experiments in which spinach was fumigated with H_2S: in these plants, the foliar cysteine pool was dramatically increased and light was required for the resultant enhancement of glutathione contents (Buwalda et al. 1988). In the dark, γ-EC accumulated. The general relevance of these observations was highlighted by the report of strong light-dependence of glutathione accumulation in poplars overexpressing γ-ECS in the cytosol (Noctor et al. 1997a). In both poplars and spinach, supplying glycine via the petiole mimicked the effect of light, causing γ-EC to remain low and glutathione accumulation to be maximized (Buwalda et al. 1990, Noctor et al. 1997b). Strong evidence that photorespiration was the light-dependent source of glycine came from studies of the effect of high CO_2 or low O_2, conditions which attenuated the effect of light (Noctor et al. 1997a, 1999).

During photorespiration, glycine is produced in the peroxisomes and, for the most part, metabolized in the mitochondria, presumably necessitating diffusion through the cytosol. The studies with poplars overexpressing γ-ECS in the cytosol therefore provided evidence that a portion of this glycine can be used in cytosolic biosyntheses, underlining the flexibility of the photorespiratory pathway (Noctor et al. 1997a,b). This flexibility, and the intracellular mobility of glycine, was further highlighted by the observation that maximal glutathione accumulation also required photorespiration in poplars overexpressing γ-ECS in the chloroplast (Noctor et al. 1999).

In untransformed poplars, the light-dependence of glutathione accumulation is much less evident than in the transformants, but it can be observed when cysteine is fed (Figure 5). The conclusion from the studies in spinach and poplar must be that the requirements of glutathione synthesis for photorespiration are generally weak, but become more significant when γ-EC synthesis is increased, to avoid accumulation of γ-EC and to sustain its efficient conversion to GSH. Photorespiration may therefore be important in supplying glycine when glutathione synthesis is up-regulated by various stress conditions. Aside from the importance of maintaining high GSH contents, it is conceivable that under some conditions, a sufficient glycine supply may be necessary to prevent accumulation of γ-EC and its possible interference in reactions such as protein thiolation.

The demands of foliar glutathione synthesis can probably be met by low rates of photorespiration (Noctor et al. 1998b), but those of non-photosynthetic tissues must be met by alternative routes (Ireland and Hiltz 1990). A possible route in tissues where the glyoxylate cycle occurs is via transamination of glyoxylate. Glycine may be produced from serine by serine hydroxymethyl transferase activity, which is found in several subcellular compartments (Besson et al. 1995) and whose activity is comparable in leaves and roots (Ireland and Hiltz 1990), though the identity of methyl group acceptors during the conversion of serine to glycine remains unclear. Glutathione production in C_4 plants such as maize is particularly interesting. Although C_4 plants probably show low but significant rates of photorespiratory glycine production (Lacuesta et al. 1997), this is confined to the bundle sheath cells, where Rubisco is located. Since glutathione synthesis is located exclusively or predominantly in the mesophyll cells, glycine may be shuttled from bundle sheath to mesophyll in the same way as proposed for cysteine.

Glutathione synthesis may also draw upon pools of other amino acids involved in the photorespiratory pathway since glutamate and serine (the precursor of cysteine: Figure 5) are involved in photorespiration. While considerable attention has been focused on relations between glutathione synthesis and S assimilation, little has been paid to interactions with N assimilation. At concentrations of up to 5 mM, glutathione offers a concentrated store of

sulphydryl groups, compared to the smaller concentrations of reductive proteins such as thioredoxins or peroxiredoxins. While the concentration of glutathione is high, its demands on primary assimilatory metabolism are comparatively low, because of relatively slow turnover rates. These may be increased by exposure to heavy metals or herbicides. Increases in glutamine synthetase have been observed on exposure of maize to cadmium (Ju et al. 1997) while, in both maize and poplar, cadmium treatment led to increased activities of enzymes involved in the production of C skeletons for N assimilation (Ju et al. 1997, Arisi et al. 2000).

Several enzymes of the ascorbate-glutathione pathway occur in glyoxysomes and peroxisomes, including APX, monodehydroascorbate reductase, and GR (Yamaguchi et al. 1995, Bunkelmann and Trelease 1996, Jiménez et al. 1997, Zhang et al. 1997b). While a putative peroxisomal gene sequence has been isolated for APX (Zhang et al. 1997b), there are as yet no candidate genes for GR specific to this compartment. Although the contribution of the ascorbate-glutathione pathway to photorespiratory H_2O_2 processing must, in most conditions, be slight compared to that of catalase, it may be crucial. Catalase has a very high maximal activity, but its affinity for H_2O_2 is much lower than that of APX. The ascorbate-glutathione pathway may therefore be required to maintain a lower peroxisomal H_2O_2 concentration than would be possible if catalase were operating alone.

Even if the ascorbate-glutathione cycle plays an important role in the peroxisomes, work with catalase-deficient plants has shown that it cannot replace catalase under conditions favourable for photorespiration. In both catalase-deficient barley mutants and tobacco in which the enzyme activity has been decreased through antisense technology, conditions favouring photorespiration were found to induce necrotic lesions and to mimic effects produced following pathogen attack (Kendall et al. 1983, Chamnongpol et al. 1996, 1998, Willekens et al. 1997, Takahashi et al. 1997, Brisson et al. 1998). Both types of plant showed a marked accumulation of glutathione when plants were transferred to 'photorespiratory' conditions (Smith et al. 1984, Willekens et al. 1997). Similar accumulation of glutathione has been observed when leaves are treated with catalase inhibitors (Smith 1985, Amory et al. 1992). These effects may have considerable physiological relevance since catalase undergoes continuous turnover in the light. Ongoing protein synthesis is required to maintain catalase activities: under conditions where degradation exceeds re-synthesis, catalase activities may decrease (Feierabend and Engel 1986). In stress conditions which impair protein synthesis, such as low or high temperature, or salt stress, a light-dependent decrease in total catalase protein and activity is observed (Volk and Feierabend 1989, Hertwig et al. 1992) and this can be accompanied by higher glutathione contents (Volk and Feierabend 1989).

A striking feature of catalase deficiency is that H_2O_2 does not accumulate. Rather, components such as glutathione appear to act as sacrificial electron donors. The mechanisms that link catalase deficiency to glutathione accumulation and oxidation are unclear. In the barley mutant, which had less than 10 % of wild-type catalase activity, foliar glutathione contents increased about 6-fold within four days of exposure to air (Smith et al. 1984). A strikingly similar accumulation of glutathione was observed within two days when antisense tobacco plants, containing about 10 % of the enzyme activity of the control tobacco, were transferred from low to moderate irradiance (Willekens et al. 1997). It is noteworthy that the increase in glutathione was specific and did not reflect a general up-regulation of soluble antioxidants (Willekens et al. 1997). The factors have not been identified that cause these marked increases in glutathione when the cell's capacity to process H_2O_2 is outstripped by the rate of photorespiratory H_2O_2 generation. One possibility is oxidation-induced de-repression of translation of the enzymes of glutathione synthesis (Xiang and Oliver 1998).

The increases in glutathione were accompanied by a marked oxidation of the glutathione pool (Smith et al. 1984, Willekens et al. 1997, Noctor et al. 2000). Again, this effect was specific and did not reflect general oxidation of cellular components, since the foliar ascorbate pool remained highly reduced in both tobacco and barley (Willekens et al. 1997, Noctor et al. 2000). Non-aqueous fractionation of leaves from the barley mutant showed that the induced glutathione synthesis and oxidation occurred in both the chloroplast and cytosol (Smith et al. 1985). Severe oxidation of the glutathione pool is unlikely to be due to direct reaction with H_2O_2, since enzymes that catalyse this reaction are very low in activity. It is also unlikely to result simply from a limiting GR activity when glutathione concentration is high. In untransformed poplars under unstressed conditions, foliar GR activity was sufficient to maintain glutathione redox state similar to untransformed controls, even when glutathione contents were markedly enhanced by chloroplastic or cytosolic up-regulation of biosynthetic capacity (Figure 4, though cf. results of Creissen et al. 1999 in tobacco). Increased oxidation of glutathione may be explained by oxidation by lipid peroxides, catalysed by GPX. More significant is likely to be increased processing of photorespiratory H_2O_2 by APX activity. In the absence of sufficient catalase activity, the accelerated rate of ascorbate oxidation may lead to net drainage of electrons from the glutathione pool. Electron flow from GSH to dehydroascorbate can occur chemically at relatively high rates and is catalysed by several different classes of proteins (Foyer and Mullineaux 1998). The specific increased oxidation state of glutathione presumably reflects an inability of GSSG reduction to keep pace with its rate of formation when flux through the ascorbate-glutathione pathway is enhanced (Noctor et al. 2000). The differences

in the responses of the ascorbate and glutathione pools may reflect the very different stability of their oxidized forms, dehydroascorbate being rapidly degraded if not re-reduced while GSSG, in contrast, has high stability.

The multiple roles of GSH within the cell, together with the stability of GSSG, may make this redox couple ideally suited to information transduction. The redox state of the glutathione pool is likely to be far more influential than its absolute size, particularly given the latter's dependence on nutrition and its role as a form of sulphur transport and storage. It is apparent that the symptoms that are observed in catalase-deficient plants under photorespiratory conditions, do not simply result from chemical damage. Rather, they resemble a precocious senescence, a regulated shutdown of leaf cell function that involves components common to signal transduction in response to pathogen attack (Chamnongpol et al. 1996, 1998, Takahashi et al. 1997, Du and Klessig 1997). A central player is salicylic acid, but disruption of glutathione homeostasis may also be a vital piece of the signalling jigsaw (Figure 1).

GLUTATHIONE HOMEOSTASIS

Maintenance of homeostasis in the face of all the metabolic demands placed on the glutathione pool involves a complex interplay between synthesis, degradation, transport, storage, oxidation-reduction, further metabolism and catabolism (Figure 7) as plants respond to environmental, developmental and nutritional cues. A complex control network regulates glutathione biosynthesis and homeostasis in plant cells. This includes coarse regulation of *de novo* synthesis of the enzymes of GSH biosynthesis and turnover and fine regulation of the flux capacity of the pathway by feedback inhibition.

While the last few years have seen considerable advances in our understanding of the molecular and metabolic control of glutathione biosynthesis in plants, less is known about the catabolism of glutathione and its homologues. Three routes of catabolism, involving GSH, GSSG and glutathione *S*-conjugates, are possible and each pathway may fulfil an essentially different function. Catabolic destruction of GSSG may serve as a detoxification process. GSSG is involved in thiolation reactions forming mixed disulphides with proteins in conditions of oxidative stress. Since this process inactivates many biosynthetic enzymes the presence of a large GSSG pool is not compatible with many metabolic reactions; catabolism of GSSG would essentially return the system to prestress homeostatis. Catabolism of GSH, on the other hand, largely concerns the remobilization of cysteine, for example during seed storage protein synthesis or during periods of sulphur deprivation. This requires the successive breakage of the two peptide bonds.

Catabolism of GSH is well-characterized in animals, failure of this process resulting in death (Meister 1998, Lieberman 1996). Transpeptidases (TPases; E.C.2.3.2.2.), which catalyse the reversible hydrolysis of the N-terminal peptide bond, initiate catabolism by removing the γ-linked glutamic acid from GSH, GSSG, glutathione conjugates and other peptides. The glutamyl moiety is either hydrolysed or donated to an amino acid acceptor or even to another GSH molecule. The second step in catabolism is less well characterized. The cys-gly bond is not unique to the glutathione tripeptide and several enzymes, including aminopeptidase M and cys-gly dipeptidase, are able to hydrolyse the bond (Meister 1988). The TPases are part of the γ-glutamyl cycle and as such are involved in amino acid transporters in some tissues (Tate and Meister 1985).

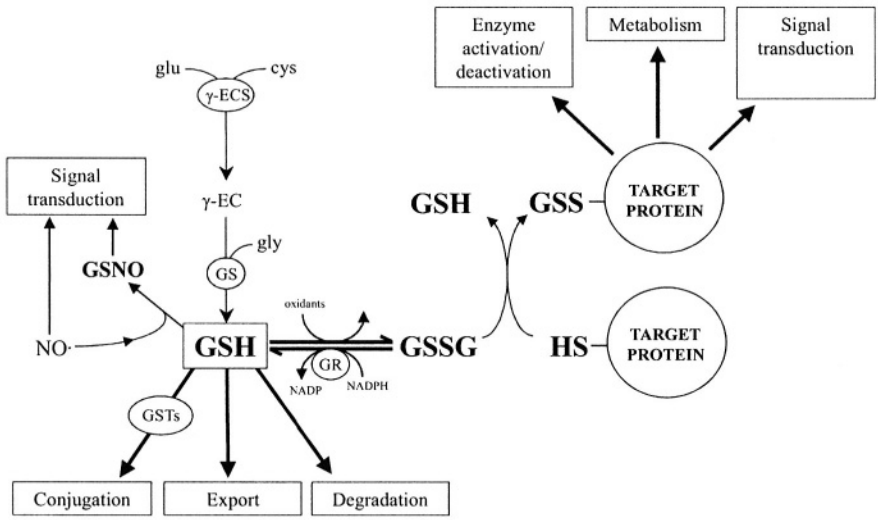

Figure 7. Processes affecting homeostasis of glutathione in plant cells and interacting processes.

The γ-glutamyl-amino acids are metabolized by a γ-glutamylcyclotransferase to oxo-prolines, which are subsequently converted to glutamate by oxo-prolinase. Homologues of these animal enzymes are also present in plants. In addition, however, a carboxypeptidase exists which is able to remove glycine as the first step of degradation, leaving γ-EC. The relative significance of these two pathways is discussed in the preceding chapter. It should be noted that a vacuolar carboxypeptidase has been identified that cleaves the glycine moiety from glutathione *S*-conjugates (Wolf et al. 1996) and that cleavage of conjugated glutathione may be a major route of catabolism. The failure to detect significant accumulation of glutathione *S*-conjugates in vacuoles suggests that they are rapidly catabolized in this compartment (Marrs 1996).

The thiol moiety of glutathione can undergo successive rounds of oxidation and reduction, without destruction of the molecule. It is clear that redox cycling of glutathione occurs at much faster rates than either synthesis or degradation. For instance, the extractable foliar activity of GR is typically 100-500 times higher than that of glutathione synthetase. This is because it is essential that sufficient amounts of reduced glutathione and high GSH:GSSG ratios are present for glutathione to fulfil its roles in metabolism and defence. In the absence of GR, the glutathione pool is lost (Kunert et al. 1990). Estimated glutathione concentrations in the chloroplast stroma range from 3.5 to 20 mM (Kunert and Foyer 1993, Noctor and Foyer 1998). Halliwell and Foyer (1978) suggested that a major function of GSH and GR in chloroplasts is to stabilize the activity of the Benson Calvin cycle. While GSH cannot replace thioredoxin in the reduction of vicinal dithiols required for the light activation of the enzymes of the Benson-Calvin cycle, a high GSH:GSSG ratio in the chloroplast stroma is crucial for their continued function (Wolosiuk and Buchanan 1977). The activities of certain of the thioredoxin-regulated chloroplast enzymes can be modulated by the GSH:GSSG ratio (Ocheretina and Scheibe 1994). Direct GSSG-dependent inactivation and GSH-mediated activation of malate dehydrogenase (Vivekanandan and Edwards 1987) and 2-carboxy-D-arabinitol 1-phosphate (CA1P) phosphatase (Heo and Holbrook 1999) has been demonstrated. In contrast, the activities of other enzymes such as glucose 6-phosphate dehydrogenase and NADP-malate dehydrogenase, are activated by oxidative stress. Changes in oxidative status may therefore influence the balance between assimilatory and dissimilatory metabolism (Kaiser 1979).

Low GSH: GSSG ratios cause inactivation of enzymes such as the chloroplastic fructose-1, 6-bisphosphatase and CA1P phosphatase, leading to the inhibition of carbon assimilation. Many groups as well as our own have demonstrated that the chloroplast GSH:GSSG ratios are high in both light and dark (Halliwell and Foyer 1978, Noctor et al. 1998b, Noctor and Foyer 1998). A recent study on *Phaseolus vulgaris,* which accumulates homoglutathione rather than glutathione, however, has shown that some oxidation of the homoglutathione pool can occur in darkness. The decrease in the hGSH:hGSSG ratio was accompanied by a large decrease in the activity of CA1P phosphatase activity and a build-up of CA1P sufficient to inhibit Rubisco (Heo and Holbrook 1999). This result is surprising but it is possible that GR has a higher affinity for GSSG than hGSSG and is unable to maintain high hGSH:hGSSG ratios *Phaseolus* leaves in the dark. It is interesting to speculate on the function of the formation of mixed disulphides between GSSG and the thiol groups of proteins. It has been known for a long time that the oxidation of critical cysteines on proteins makes them targets for proteolysis. Thiolation, under conditions of oxidative stress, would inactivate

some chloroplastic enzymes and activate others but would also simultaneously protect them from proteolytic attack.

Tissue GSH:GSSG ratios have been implicated in various signal transduction processes that are crucial to plant defence responses. These ratios are involved in the control of the expression of defence genes, as discussed earlier. In addition, there is evidence that GSH is involved in the regulation of cell division (Sanchez-Fernandez et al. 1997, May et al. 1998a). Crucial to signal transduction processes associated with defence responses appears to be the interaction between SA, hydrogen peroxide and glutathione (Rao and Davis 1999). Perturbation of the balance between these components may have led to the chlorotic phenotypes observed in transformed tobacco overexpressing γ-ECS in the chloroplasts (Creissen et al. 1999).

The redox state of the glutathione pool is remarkably constant but extreme oxidative stress leads to oxidation of the pool, as observed in plants with low catalase activities and during the hypersensitive response (Smith et al. 1984, Willekens et al. 1997, Vanacker et al. 2000). In plants, as in animals, cell growth and death responses appear to be coupled. Morphogenesis is achieved through differential cell division and cell expansion in response to positional cues generated during development by cell-to-cell communication. At maturity, tissue homeostasis can be influenced by the action of growth regulators, which act singly or in combination. Growth and development are also influenced by cell death, which is required for processes such as the formation of tracheary elements, the release of mature pollen, the selective elimination of organs during embryogenesis and, in some species, during flower development. Damaged cells that are not eliminated during PCD can proliferate and form tumours. Glutathione has been found to be necessary for the cell to enter the G_1 phase, the pre-mitotic phase of the cell cycle in which the cell is capable of responding to extracellular stimuli that determine whether it will enter the S phase, or enter quiescence, differentiation or death (May et al. 1998a).

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Chapter 4

THE ROLE OF GLUTATHIONE IN THE UPTAKE AND METABOLISM OF SULFUR AND SELENIUM

John W. Anderson and Peter J. McMahon

INTRODUCTION

Sulphur (S) is essential for plant growth since it is a constituent of the two S-amino acids, cysteine and methionine, which occur in protein. S also occurs in plants in various compounds of low molecular weight, many of which are secondary compounds. They include the glucosinolates found in many *Brassica* species and the non-protein S-amino acids found in most *Allium* species. In some species these can be quantitatively important sources of S. In general, however, the abundance of S in plants is linked to the abundance of N in protein which contains both N and S. It therefore follows that one of the main determinants of the N:S ratio in plants is the frequency of the protein S-amino acids relative to the total number of protein amino acids. In vegetative plants, the N:S ratio is determined primarily by the amino acid sequence of the individual constitutive proteins and the abundance of each protein. The proteins of vegetative cereals are thought to have a N:S ratio (expressed on a molar basis) of about 30 (Djikshoorn and van Wijk 1967, Bolton et al. 1976). On the other hand, the molar N:S ratio of generative tissue depends on the frequency of the S-amino acids in the various storage proteins and the relative amounts of each protein that are made. This can be very variable as it depends on the level of N and S nutrition.

Plants, unlike animals, acquire their S as sulphate from soil via an energy-requiring sulphate-specific carrier mechanism. The activity of the uptake mechanism is tightly controlled. Plants also regulate the rate at which they assimilate sulphate-S into organic S. Further, since almost all of the organic S in plants occurs in S-amino acids, then the acquisition and assimilation of inorganic S must be coordinated with the acquisition and availability of inorganic N. Thus, plants must have mechanisms for sensing the N status of the plant and using this information to adjust the rate of S acquisi-

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tion. In this way, the relative availability of N and S is maintained at levels, which represent their abundance in the proteins of plant material. It follows that plants must also have some way of sensing the S status of cytoplasm and using this information as a signal to regulate the activity of the S uptake mechanism; a high or adequate internal S status inhibits or down-regulates the uptake mechanism and low internal S enhances sulphate uptake. Glutathione, together with cysteine and sulphate, is regarded as an important indicator of the internal S status of plants though, of these, only free cysteine and glutathione appear to comprise the primary S-sensing pool for regulating S uptake and S assimilation. It is likely that the effect of glutathione is mediated indirectly via cysteine metabolism.

This chapter is mostly concerned with three issues. The first concerns the indirect or regulatory role of glutathione in the uptake and assimilation of sulphate-S into organic S by plants. If sulphate uptake and assimilation are regulated via changes in the intracellular concentration of glutathione, this hypothesis can be tested by examining net changes to the glutathione pool in response to certain events and the consequences that this has with respect to sulphate uptake and assimilation. The second issue involves the role of glutathione in the long distance transport of organic S in vegetative and generative plants and its role in signalling the S status/demand between the various plant parts. The third concerns the role of glutathione in the reductive assimilation of inorganic selenium (Se).

SULPHATE UPTAKE MECHANISMS AND THEIR REGULATION

Under normal conditions, plants grown in aerobic soils in unpolluted air acquire their S predominantly via the root system as inorganic sulphate. The kinetics of sulphate uptake by plant roots are biphasic and this has long been taken to imply the existence of low and high affinity uptake mechanisms (for a review of earlier literature, see Anderson 1990). The high affinity mechanism in root parenchyma cells is driven by a proton pumping ATPase with the export of $3H^+$ per sulphate imported implying an energy cost of 3ATP per sulphate (Clarkson 1985). This activity is strongly enhanced (up to 10-fold) by a short period of S starvation (Clarkson et al. 1983, Hawkesford and Belcher 1991).

Recently, much progress has been made in the study of sulphate transporting systems in plants particularly in the tropical pasture legume, stylo (*Stylosanthes hamata*), but also in other species (Takahashi et al. 1996, Hawkesford and Smith 1997). In stylo, the enhanced uptake of sulphate following termination of the sulphate supply is associated with enhanced ex-

pression of two sulphate uptake transporting proteins (SHST1 and SHST2) with high affinity for sulphate (K_m *ca* 10 μM). On the basis of their homology with various other transport proteins, they are most likely associated with the plasma membrane and are therefore probably not involved in intracellular sulphate transport (Hawkesford and Smith 1997). The expression of these transporters in turn can be traced to transcriptional control of the genes *shst1* and *shst2* (Hawkesford and Smith 1997). This chain of events is readily reversed by addition of sulphate, indicative of repression either by sulphate itself or by a product formed from it (Clarkson et al. 1983). The decline in uptake activity is very rapid (half time of several hours) indicating that the sulphate transporting proteins and the associated mRNAs are subject to rapid turnover. A third sulphate transporter SHST3 has also been detected in *S. hamata* and appears to have less in common with SHST1 and SHST2; it has a truncated amino acid sequence at the N-terminal end and a much lower affinity for sulphate (K_m *ca* 100 μM). Northern blot analyses indicate that the mRNAs for *shst1* and *shst2* are expressed exclusively in root tissue and their levels are rapidly enhanced when the sulphate supply is terminated. These features suggest that the high affinity transporters SHST1 and SHST2 are involved in the acquisition of exogenous sulphate from the root environment. Fewer details are available for the expression of *shst3* but it appears to be expressed at lower levels in both root and shoot tissue and it does not appear to respond to sulphate deprivation in the same way as *shst1* and *shst2* (Hawkesford and Smith 1997). A point of interest here is that most plant organs are involved at some stage of development with loading and/or unloading sulphate from the phloem, raising the possibility that SHST3 could be involved in this activity.

The decrease in sulphate uptake activity that follows the application of sulphate to S-deprived plants is preceded by significant increases in the internal pools of sulphate, cysteine and glutathione (Brunold 1993, Kredich 1993, Herschbach and Rennenberg 1994). Logically, a decrease in the concentration of one or more of these metabolites could act as the signal for derepression of sulphate uptake in S-starved plants. Since sulphate is abundant in the vacuoles of cells and vacuolar sulphate is in passive equilibrium with sulphate in the cytoplasm (Cram 1983a,b), sulphate is regarded as an unlikely candidate for directing the activity of the sulphate uptake mechanism. Moreover, sulphate itself is the immediate uptake product. On the other hand, cysteine and glutathione are products of sulphate assimilation and their production is linked to many cellular activities making them more relevant as regulatory sensors.

The synthesis of glutathione responds rapidly to various environmental stimuli. Since glutathione is synthesized from cysteine, then sulphate uptake, the assimilation of sulphate into cysteine, and the synthesis of glutathione

from cysteine must all respond to the increased demand for S for glutathione synthesis. The cytoplasmic concentration of cysteine is maintained within a tight range and the flux of S from cysteine into glutathione appears to be regulated by the internal concentration of glutathione itself. Thus, the most likely candidate for direct negative control of sulphate uptake would appear to be cysteine. In this event, control of sulphate uptake for glutathione synthesis would be mediated indirectly by glutathione through the demand for cysteine.

Plants appear to have additional mechanisms to keep sulphate uptake activity in tune with their S requirements. Smith (1980) first reported that the concentration of *O*-acetylserine (OAS) in cultured tobacco cells increased when the cells were starved of sulphate, raising the possibility that OAS could be an important positive signal for promoting sulphate uptake. More recently it has been established that when OAS is supplied in the growing medium to plants grown with adequate S, the internal concentrations of glutathione and cysteine increase substantially (Hawkesford and Smith 1997). Thus, it appears that OAS can act as a positive regulator of sulphate uptake and countermand the negative effects of cysteine/glutathione which tend to decrease the flux of S from sulphate into the internal cysteine and glutathione pools. The concentration of OAS may also be important in the coordination of N and S assimilation.

ASSIMILATION OF SULPHATE-S INTO GLUTATHIONE

The assimilation of sulphate into glutathione involves two interlinked pathways (Figure 1). The first involves the assimilation of sulphate into cysteine (Figure 1, reactions 1-4). This entails the activation of sulphate by ATP in a reaction catalysed by ATP sulphurylase to form adenosylphosphosulphate (APS) with the elimination of pyrophosphate (PPi) and the subsequent transfer of the sulphonyl moiety to an endogenous compound of unknown composition (carrier-SH) in a reaction catalysed by APS sulphotransferase. The bound sulphonyl group is then reduced by ferredoxin (reduced form) in a reaction involving organic thiosulphate reductase producing bound sulphide. Finally bound sulphide is incorporated into OAS to form cysteine with the elimination of acetate in a reaction catalysed by cysteine synthase (also known as OAS (thiol) lyase). The sulphide acceptor, OAS, is formed from serine and acetyl-CoA in a reaction catalysed by serine transacetylase. This reaction (Figure 1, reaction 7) is of considerable importance with respect to S assimilation since OAS is potentially important in coordinating N and S assimilation and, as already noted, OAS appears to be important in

effecting positive control over sulphate uptake. Plants also have a very active pathway for the reductive assimilation of free inorganic sulphite into cysteine in chloroplasts involving sulphite reductase (Ng and Anderson 1979). This pathway, which does not act on sulphate, is thought to be involved in the detoxification of SO_2 acquired from polluted air (Anderson 1990, De Kok 1990).

The chloroplasts of expanding leaves are the primary site for the reductive assimilation of sulphate into cysteine. Sulphate assimilation in chloroplasts is strongly light-coupled (for review, see Anderson 1990) indicating that illuminated chloroplasts use ATP and reduced ferredoxin from the light reactions to drive the pathway (Figure 1) in much the same way as light drives the reductive assimilation of CO_2 and inorganic N. Thus, in leaves, the reactions shown for cysteine synthesis in Figure 1 can be regarded as the dark reactions of reductive sulphate assimilation.

Since sulphate is acquired mostly from soil, sulphate transport from the root to the site of sulphate assimilation in young expanding leaves comprises an essential part of sulphate assimilation in whole plants. Non-photosynthetic tissues, including root tissue, also contain the enzymes for reductive sulphate assimilation (Anderson 1980, 1990). However, their activity is regarded as quantitatively unimportant under normal conditions.

Although cysteine is regarded as the immediate end-product of sulphate assimilation (Figure 1, reactions 1-4), the concentration of free cysteine in plants is normally very low and is maintained within tight limits except in tissues treated with gaseous SO_2 and H_2S (Rennenberg 1984, De Kok 1990). In view of this and the fact that the pathway for cysteine synthesis (Figure 1) accounts for all of the S assimilated by plants under normal conditions, the pathway must be very responsive to changes in the demand for cysteine for the various other pathways that it supplies. The metabolism of cysteine to glutathione, the second component pathway in the assimilation of sulphate into glutathione (Figure 1, reactions 5-6), is just one of several pathways (albeit an important one) that draws upon the pool of endogenous cysteine. Specifically, glutathione synthesis involves coupling of cysteine with glutamate in an ATP-requiring reaction to form γ-glutamylcysteine catalysed by γ-glutamylcysteine synthetase and addition of glycine in another ATP-requiring reaction catalysed by glutathione synthetase (Noctor and Foyer 1998, Noctor et al. 1998).

Currently available evidence indicates that there is no one specific intracellular site for glutathione synthesis in plant cells (for reviews, see Rennenberg 1997, Noctor and Foyer 1998). The general view is that glutathione synthesis occurs in both the cytosol and in chloroplasts. Here it is pertinent to consider the pools of the relevant amino acids, particularly cysteine, that are available for recruitment into glutathione. In this regard the chloroplast is

a rich source and is the primary site of assimilatory sulphate reduction. Presumably ATP formed in the light can be used to support the reactions catalysed by γ-glutamylcysteine synthetase and glutathione synthetase (Figure 1, reactions 5-6) in chloroplasts in the same way as the products of the light

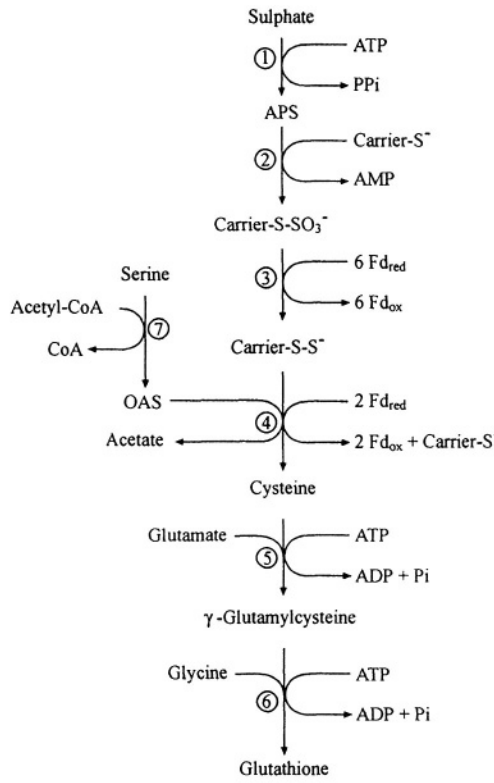

Figure 1. Pathway for the flow of S from sulphate into glutathione. Reactions 1-4 comprise assimilatory sulphate reduction and result in the synthesis of cysteine. The enzymes supporting these reactions are most active in the chloroplasts of young expanding leaves. Reactions 5-6 involve the synthesis of glutathione from cysteine. The enzymes supporting these reactions are located in both chloroplasts and in the cytosol. Reaction 7 involves the production of *O*-acetylserine (OAS), which acts as the sulphide acceptor for cysteine synthesis. Chloroplasts also have the capacity to reductively assimilate free sulphite into cysteine via sulphite reductase activity but this is only thought to be important when plants are exposed to air containing gaseous S pollutants. In some species, phosphoadenosylphosphosulphate (PAPS) rather than adenosylphosphosulphate (APS) is regarded as the physiologically important substrate for sulphate for reduction. Enzymes involved in the pathway are: 1, ATP sulphurylase; 2, APS sulphotransferase, 3, organic thiosulphate reductase; 4, cysteine synthase; 5, γ-glutamylcysteine synthetase; 6, glutathione synthetase; 7, serine transacetylase.

reaction support cysteine synthesis (Figure 1, reactions 1-4) and thereby account for the glutathione that occurs in chloroplasts. A possibility to consider here is that the starting materials for glutathione synthesis could be quite different in the chloroplast and in the cytosol: in chloroplasts, the starting source is likely to include the cysteine formed *de novo* from newly assimilated sulphate whereas at least some of the cysteine used for glutathione synthesis in the cytosol is likely to have quite a different origin, especially in mature or senescing cells or cells undergoing protein mobilization.

Glutathione synthesis in the cytosol does not appear to have been placed in physiological perspective. There is growing evidence that N-stressed senescing leaves and the endosperm of germinating cereal seeds are active sites of glutathione synthesis (Sunarpi and Anderson 1997a,b, Imsic and Anderson unpublished). Since, at least in germinating seeds, chloroplasts cannot be involved it would seem likely that the cysteine required for glutathione synthesis is derived by hydrolysis of proteins in the cytosol/protein bodies in these tissues/organs. Logically, this would involve protein hydrolysis to release free cysteine and methionine, metabolism of methionine to cysteine, and incorporation of cysteine into glutathione (Figure 2); it would entail a very different complement of enzymes to those required for the postulated synthesis of glutathione from sulphate in chloroplasts. Of particular interest is the synthesis of cysteine from methionine, a process which in theory (Giovanelli et al. 1980) would involve catabolism of cystathionine via γ-cystathionase activity (Figure 2, reaction 4) rather than the anabolic enzyme β-cystathionase required for methionine synthesis.

An issue closely linked to the question of the sites of glutathione synthesis is the permeability of chloroplasts to glutathione and the concentration of glutathione in the various intracellular compartments. The concentration in chloroplasts is considerably higher than the average concentration of mesophyll cells (Foyer and Halliwell 1976, Law et al. 1983). Largely for this reason, it has often been assumed that the chloroplast is impermeable to glutathione, a view supported by the observation that lysed chloroplasts but not intact chloroplasts exhibit light-dependent reduction of exogenous oxidized glutathione (Jablonski and Anderson 1978, Anderson et al. 1983). However, little is known about intracellular transport of glutathione in plant cells and the concentration of glutathione in individual metabolic compartments, especially the concentration in the cytosol (Noctor and Foyer 1998). Nonetheless, there are important reasons for suspecting that plant cells have mechanisms for the transport of the tripeptide glutathione across the plasma membrane. In particular, glutathione occurs in phloem and is regarded as an important transport compound , implying a phloem loading mechanism for this metabolite. Also, the grains of generative cereals recruit glutathione from the endosperm cavity . There is now strong support for the existence of a glu-

tathione transport mechanism in the plasma membrane (Schneider et al. 1992, Jamai et al. 1996); the transport mechanism in broad bean leaf protoplasts appears to be able to transport both GSSG and GSH in association with proton symport (Jamai et al. 1996). In this regard, it is interesting to note that a peptide transport protein has been reported in *Arabidopsis* (Steiner et al. 1994) but the specificity with respect to glutathione does not appear to have been examined.

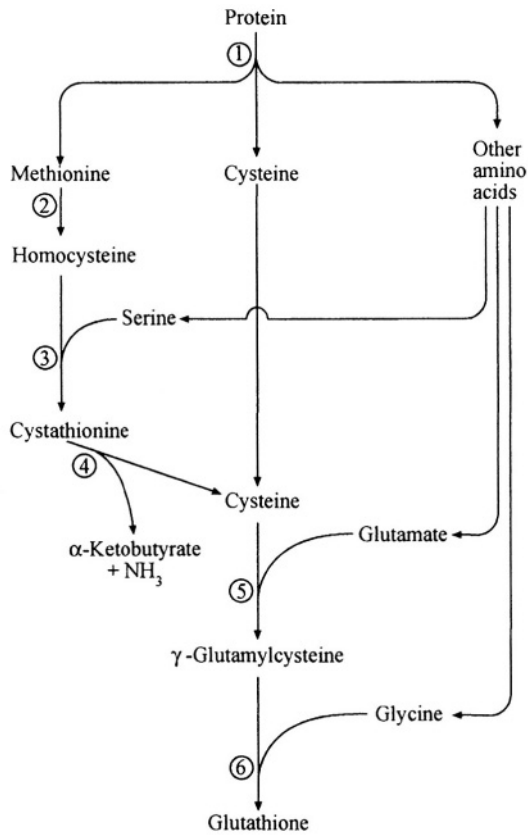

Figure 2. Postulated scheme for the synthesis of glutathione from protein. The scheme would be most likely to be active in senescing tissues or tissues engaged in protein mobilization. Enzymes involved in the pathway are: 1, various proteases and peptidases; 2, methionine demethylation, perhaps involving *S*-adenosylmethionine as an intermediate; 3, cystathionine β-synthase; 4, γ-cystathionase; 5, γ-glutamylcysteine synthetase; 6, glutathione synthetase.

PERTURBATION OF THE INTRACELLULAR CONCENTRATION OF GLUTATHIONE

A number of environmental and physiological factors cause changes in the endogenous glutathione concentration, some positive, some negative. Factors that serve to decrease the concentration of glutathione include increased demand to support growth, production of phytochelatins in response to heavy metals, detoxification of xenobiotics, sequestration of S in secondary metabolites, glutathione export (a process involving long distance transport to sites of high demand for organic S such as developing grains in generative plants) and S starvation (for reviews, see Rennenberg and Lamoureux 1990, Bergmann and Rennenberg 1993). Conversely, high levels of S nutrition or the removal of a negative influence serve to enhance the concentration of glutathione.

Physiologically, changes in the concentration of glutathione in plant tissues must involve variations in input (biosynthesis, import) and/or output (catabolism, export, metabolic demand). For example, when plants are exposed to cadmium (Cd) they consume glutathione to synthesize a family of peptides known as phytochelatins (see below) thereby causing a short-term decrease in the tissue concentration of glutathione. Some of the activities listed in Figure 3 also tend to cause short-term depletion of the glutathione pool. Conversely, the concentration of cysteine in plants is normally very low and appears to be under tight regulation. Given this, it follows that any one of the factors that effect changes in the glutathione pool (Table 1) has the potential to influence sulphate uptake and cysteine synthesis. Further, since plants contain mechanisms for the interconversion of cysteine and glutathione, Rennenberg (1982, 1984) has proposed that glutathione plays an important role in mantaining the low homeostatic concentrations of cysteine common to all plants so that glutathione effectively serves as a strategic reserve of cysteine for the synthesis of other organic-S compounds.

Reports of glutathione perturbations such as those reported in Table 1 are often confused by technical issues. It is not uncommon to measure only the reduced form of glutathione (GSH) in plant extracts. As GSH is normally far in excess of the oxidized form (GSSG) (Smith et al. 1985), changes in GSH usually reflect changes in total glutathione (i.e. GSH + GSSG). However, factors that cause oxidative stress decrease the GSH/GSSG ratio so that care is required when interpreting reports of perturbations of glutathione pools based on measurements of GSH. Studies in which a reducing agent is used to convert GSSG to GSH prior to analysis (e.g. Klapheck et al. 1992, Table 1) provide data on the total glutathione concentration.

Table 1. Effect of environmental and stress factors on the concentration of glutathione and other S-containing metabolites in plants. Responses are shown as an increase (+) or as a decrease (–) in the concentration of the relevant metabolite. Abbreviations: AOS, active oxygen species; Cys, cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; hGSH, homoglutathione; hm-GSH, hydroxymethylglutathione; Met, methionine.

Factor	Response	Plant/tissue	Reference
Limited S	– GSH, Cys, Met	Tobacco cells	Smith 1980
High S (sulphate)	+ GSH	Spinach leaves*	De Kok and Kuiper 1986
	+ GSH, hm-GSH	Wheat leaves*	Klapheck et al. 1992
High S (gaseous)	+ GSH, GSSG	Spinach shoots	De Kok et al. 1986
Heat	+ GSH – Cys	Maize roots	Nieto-Sotelo and Ho 1986
Chilling	+ GSH, Cys, SO_4^{2-} assimilation	Maize roots and shoots⊕	Kocsy et al. 1996
Drought	– GSH/GSSG ratio	Moss *(Tortula)*	Dhindsa 1991
Pathogens	+ GSH, hGSH	Alfalfa cells	Edwards et al. 1991
	+ hGSH	Bean cells	Edwards et al. 1991
AOS	– GSH/GSSG ratio	Barley leaves	Smith et al. 1985
	+ GSH (– GSH/GSSG ratio)	Poplar leaves	Sen-Gupta et al. 1991
	+ GSH	*Arabidopsis* cells	May and Leaver 1993
Herbicide safeners	+ GSH, Cys + SO_4^{2-} uptake and assimilation	Maize roots	Farago and Brunold 1990
Cadmium	– GSH (short-term)	Maize roots	Meuwly and Rauser 1992
	– GSH (short-term) + SO_4^{2-} assimilation	Maize roots	Ruegsegger and Brunold 1992

* leaf sections incubated *in vitro*
⊕ chilling tolerant genotypes only

The existence of a small steady-state cysteine pool and the relatively large changes in glutathione that occur in response to certain stimuli (Table 1) implies that sulphate uptake is negatively regulated by glutathione, either directly or indirectly (Clarkson et al. 1983, Rennenberg et al. 1989, Rennenberg and Lamoureux 1990, Herschbach and Rennenberg 1991); it also implies negative regulation of sulphate assimilation into cysteine. Thus, according to this idea, factors that deplete the intracellular glutathione pool promote sulphate uptake and the assimilation of sulphate-S into cysteine and glutathione. Conversely, factors that retard or inhibit consumption/metabolism of glutathione inhibit these processes. Various observations are consistent with this general hypothesis. One of the best known examples involves the events associated with the application of cadmium (Cd) and various other heavy metals to plants. Cd elicits the production of a family of cysteine-rich peptides known as phytochelatins (PCs) with the composition

(γ-glutamyl-cysteinyl)$_n$-glycine where n is typically 2-7 (Kondo et al. 1984, Grill et al. 1985). The PCs formed in response to Cd in turn chelate with Cd to form Cd-PC complexes, which, according to current theory, are transported into vacuoles where they interact with sulphide-S to form larger Cd-PC complexes which effectively immobilize the Cd within the plant (Rauser 1990, 1995). PCs are formed from glutathione in a reaction catalysed by PC synthase according to the reaction

$$GSH + PC_n \Rightarrow PC_{n+1} + \text{glycine}$$

This response occurs rapidly following the administration of Cd in a process that reportedly involves post-transcriptional activation of PC synthase (Grill et al. 1989). Theory predicts that Cd elicits a short-term decrease in the intracellular concentration of glutathione and conversely, Cd enhances sulphate uptake and cysteine synthesis to sustain PC production. These predictions are borne out by experiment. Cd enhances the uptake of sulphate in wheat (McMahon 1998) and causes a short-term decrease and a subsequent increase in the concentration of glutathione in cell cultures and wheat roots to sub-control levels (Scheller et al. 1987, Mendum et al. 1990, McMahon 1998). The reason that the concentration of glutathione does not re-attain its initial value, particularly in roots (Meuwly and Rauser 1992, Ruegsegger and Brunold 1992) is uncertain especially as there is no evidence for Cd-impaired transport of glutathione from the shoot to the root (Rauser et al. 1991, Rauser 1993). However, it would appear to reflect a new equilibrium between glutathione input (synthesis and import) and output for PC synthesis and other activities in the root in the presence of Cd. The lower steady-state concentration of glutathione would have the effect, directly or indirectly, of alleviating feed-back repression of sulphate uptake and assimilation thereby promoting the capacity for the flow of S from sulphate via cysteine to glutathione .

The effect of Cd on endogenous glutathione appears to be interactive with the effect of S nutrition. Plants grown at low S take longer to attain a new steady state concentration of glutathione following the application of Cd and exhibit larger increases in γ-glutamylcysteine than plants grown at high S (McMahon 1998). These data are consistent with the proposal that low concentrations of glutathione alleviate both the inhibition of assimilatory sulphate reduction and the feedback inhibition of γ-glutamylcysteine synthetase.

Some xenobiotic substances, including herbicide safeners, also perturb the glutathione pool in plants. Herbicide safeners are substances that protect crop plants from herbicides by promoting the detoxification of herbicides by xenobiotic-induced formation of *S*-glutathione conjugates of the herbicide, catalysed by glutathione *S*-transferase:

Glutathione + herbicide ⇒ glutathione *S*-herbicide

This is primarily achieved by enhancing glutathione *S*-transferase activity (Dean et al. 1990, Lamoureux and Rusness 1993, Farago et al. 1994), possibly at the transcriptional level (Wiegand et al. 1986). Safeners also enhance the concentration of glutathione though this is reportedly not of primary importance in eliciting protection against the target herbicide *in vivo* (Farago et al. 1994), perhaps reflecting the relatively high affinity of glutathione *S*-transferase for glutathione (K_m 100-200 μM, Habig et al. 1974). However, safeners reportedly increase the flux of S from sulphate into glutathione (Farago and Brunold 1990, Lamoureux and Rusness 1993) consistent with the allocation of additional S into glutathione for conjugation of the herbicide.

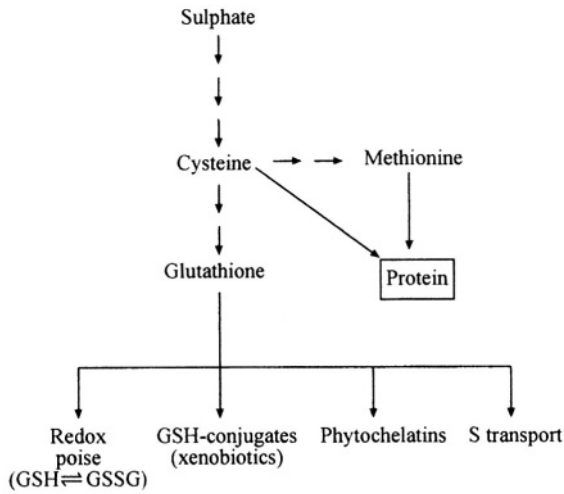

Figure 3. Summary of the processes in plants that require S, which are mediated via cysteine and glutathione.

In theory, another potential positive influence on the concentration of glutathione in cells which seems to have received little attention is the production of glutathione from the products of protein hydrolysis (Figure 2). For example germinating seeds exhibit large decreases in protein-S (Sunarpi and Anderson 1995) with a concomitant increase in glutathione. This activity also appears to be important in senescing leaves of vegetative plants grown at low N and in generative plants grown under field conditions with inadequate S (Fitzgerald et al. 1999a). Under the latter conditions, generative plants actively synthesize seed storage proteins but *de novo* uptake of exoge-

nous nutrients is quantitatively unimportant due to diminished water availability (Smith and Whitfield 1990).

The redox turnover of the oxidized and reduced forms of glutathione in chloroplasts has been extensively studied in relation to leakage of reducing equivalents into oxygen from photosystem I (for a review see Noctor and Foyer 1998). However, there seems to be little information on changes in the total glutathione pool in chloroplasts of photosynthetically active leaves. Also, the fate of glutathione in senescing chloroplasts, an issue that begs the question of the permeability of the chloroplast envelope to glutathione, does not appear to have been studied.

REGULATION OF SULPHATE ASSIMILATION AND GLUTATHIONE SYNTHESIS

All of the S required for plant growth and function must be processed via the cysteine pool, which is normally maintained at a very low concentration within a narrow range. Since glutathione is formed from cysteine, the rate of glutathione synthesis varies with many environmental and physiological factors. Therefore, plants require regulatory mechanisms to adjust the flow of S into glutathione in response to changes in demand. In view of the small cysteine pool, variations in the demand for glutathione must be linked to changes in the rate of both sulphate assimilation and the incorporation of cysteine into glutathione. This appears to involve regulation at both the transcription and the post-translational levels.

Control of assimilatory sulphate reduction

Control of S flux from sulphate to cysteine appears to be controlled in several ways (Figure 4). The first concerns the transcriptional control over the sulphate transporting membrane proteins. As discussed before,, expression of these proteins is linked to low concentrations of cysteine, glutathione and sulphate though there are reasons for suspecting that glutathione serves as the long distance signal and that cysteine acts as the principal direct sensor for initiating transcription. Conversely, high concentrations of OAS appear to enhance the expression of the sulphate transporting proteins. The activities of various enzymes of the sulphate assimilation pathway are also influenced by factors that affect glutathione synthesis. Cultured tobacco cells and plants exhibit large increases in the extractable activity of ATP sulphurylase when exposed to S stress (for a review, see Brunold 1990). S dep-

rivation influences the expression of ATP sulphurylase and APS-kinase indicating that control over sulphate assimilation occurs, at least in part, at the level of transcription (see Hell 1997). However, of the enzymes of sulphate assimilation, APS sulphotransferase seems to be especially responsive. For example, APS sulphotransferase activity increases 2-fold in response to S deprivation (Brunold et al. 1987) and up to 20-fold in plants exposed to herbicide safeners (Farago and Brunold 1990); conversely, the activity decreases on exposure to SO_2 or H_2S (Tschanz et al. 1986) and to exogenous cysteine (Jenni et al. 1980). These responses provide conditions for enhancing S flux under conditions where there is an increased demand for glutathione synthesis and suggest that control of APS sulphotransferase activity is important in assimilatory sulphate reduction. The activity of ATP sulphurylase does not appear to be especially sensitive to the demand for S (Brunold et al. 1987), consistent with the very high activity of this enzyme in plant tissues. However, ATP sulphurylase activity appears the linked to the rate of N assimilation.

Since glutathione is important in many aspects of plant function (Figure 3), Bergmann and Rennenberg (1993) have suggested that it is a likely regulatory factor in glutathione synthesis. May et al. (1998) have proposed a model for the control of sulphate assimilation by glutathione in which glutathione blocks the transduction into the nucleus of an unidentified factor which controls the expression of the proteins of the sulphate assimilation pathway. Perhaps this model might also be applicable to regulation of the sulphate transporter.

Control of glutathione synthesis from cysteine

In theory, the synthesis of glutathione can be regulated by the availability of one or more of the constituent amino acids, cysteine, glutamate and glycine, and/or the activity of one or more of the enzymes involved in coupling them (Brunold and Rennenberg 1997). Under normal conditions, glutamate and glycine do not appear to be limiting. Rather, the endogenous concentration of cysteine and the activity of γ-glutamylcysteine synthetase (and hence the concentration of γ-glutamylcysteine) appear to be critically important (Brunold and Rennenberg 1997). A significant feature is that both the K_m(cysteine) of γ-glutamylcysteine synthetase and the intracellular concentration of cysteine are very low thereby making the synthesis of γ-glutamylcysteine especially sensitive to the endogenous concentration of cysteine. In illuminated plants addition of exogenous cysteine consistently enhances the internal concentration of glutathione (Buwalda et al. 1990,

Farago and Brunold 1994, Schneider and Bergmann 1995, Noctor et al. 1996, 1997) implying that the production of glutathione is limited by the availability of γ-glutamylcysteine which in turn is limited by the internal concentration of cysteine. Other evidence is consistent with this view. In the leaves of poplar plants in which the limited production of γ-glutamylcysteine is overcome by overexpression of γ-glutamylcysteine synthetase, glutathione synthesis is limited by glutamate rather than cysteine (Noctor et al. 1996). This implies that the synthesis of cysteine from sulphate is responsive to increased demand for cysteine for glutathione synthesis. Studies of γ-glutamylcysteine synthetase activity in relation to glutathione and PC synthesis in plants and cell cultures treated with Cd are consistent with the proposals set out above.

Studies with poplar plants that overexpress glutathione synthetase indicate that the activity of this enzyme does not normally limit glutathione (Strohm et al. 1995, Brunold and Rennenberg 1997). The endogenous glutathione concentration of transgenic plants was similar to that of the wild type and addition of cysteine enhanced the endogenous glutathione concentration in both plants. However, upon addition of cysteine the concentration of glutathione did not increase with time indefinitely, either in the wild type or transgenic plants, even though they accumulated cysteine. This implies that some other factor limits the synthesis of glutathione from cysteine. This appears to involve γ-glutamylcysteine synthetase activity since addition of γ-glutamylcysteine resulted in much higher concentrations of glutathione in the plants, which overexpressed glutathione synthetase (Strohm et al. 1995). Since glutathione strongly inhibits γ-glutamylcysteine synthetase *in vitro* (Hell et al. 1990), other observations with Cd-treated cells and plants have been interpreted in terms of a model involving negative post-transcriptional control of γ-glutamylcysteine synthetase by glutathione (Ruegsegger and Brunold 1992, Bergmann and Rennenberg 1993). However, feedback control of γ-glutamylcysteine synthetase by glutathione was not evident in transformed poplar plants which overexpressed γ-glutamylcysteine synthetase even though the concentration of glutathione in these plants was 3-fold higher than in the wild type. Another relevant factor here is that, under some conditions, glutathione accumulates in response to various external factors (Table 1) indicative of lack of feedback inhibition. However, it is not known whether these concentrations apply at the subcellular sites where the regulatory mechanisms of glutathione synthesis are located.

Collectively, the available data indicate that glutathione synthetase is not an important regulatory site and that the reaction catalysed by this enzyme is close to thermodynamic equilibrium (Noctor et al. 1998). Conversely, the reaction catalysed by γ-glutamylcysteine synthetase is far removed from chemical equilibrium and appears to be regulated in several different ways,

both pre-translationally and post-translationally. In addition to *in vitro* evidence of feedback control by GSH (Hell et al. 1990), post-transcriptional control of this enzyme is supported by the poor expression of *Arabidopsis* γ-glutamylcysteine synthetase in transformed *E. coli*, leading to the suggestion that post-transcriptional factors, absent in the bacteria, are necessary for full expression of the plant gene (May et al. 1998). It appears therefore that γ-glutamylcysteine is the primary point of regulation of glutathione synthesis. It is controlled by both the availability of cysteine and by the activity of γ-glutamylcysteine synthetase. As plants that overexpress γ-glutamylcysteine synthetase appear to adjust their rate of S assimilation to support γ-glutamylcysteine (and hence glutathione) synthesis, then the availability of γ-glutamylcysteine might represent a secondary regulatory point in the pathway of glutathione synthesis. This would also prevent the concentration of glutathione exceeding a certain limit, perhaps for physiologically relevant reasons.

Under special conditions plants can produce abnormally high concentrations of cysteine. This can be achieved by fumigating plants with H_2S which becomes assimilated into cysteine (thereby acting as a detoxification mechanism) and simultaneously placing the plants in the dark to inhibit photorespiratory production of glycine. Under these conditions, the production of glutathione can be limited by the availability of glycine (Buwalda et al. 1988, 1990).

As noted before, glutathione synthesis occurs in both the chloroplast and the cytosol. This raises the question of the availability of the constituent amino acids and the permeability of the intracellular membranes to cysteine, glycine and glutamate. Since it is likely that the amino acids used in glutathione synthesis in chloroplasts and the cytosol have different origins, perhaps glutathione synthesis could be directly or indirectly regulated by intracellular amino acid transport (e.g. transport of cysteine from the primary site of synthesis in the chloroplast for use in glutathione synthesis in the cytosol, Rennenberg 1997).

Regulatory responses associated with exposure to cadmium and herbicide fasteners

Cd promotes the synthesis of PCs from glutathione. Sustained production of PCs implies increased flux of S from sulphate via cysteine into glutathione. According to the scheme given in Figure 4, the short-term decrease in the concentration of glutathione following the administration of Cd should lead to increased flux of S from sulphate into glutathione. This has

been confirmed by experiment. For example, Nussbaum et al. (1988) and Ruegsegger and Brunold (1992) reported that Cd enhanced the rate of sulphate assimilation in the root and increased the turnover of cysteine, γ-glutamylcysteine and glutathione, consistent with the proposed role of glutathione as a direct or indirect signal for controlling the flux of S from sulphate into glutathione. Cd enhanced the activities of several enzymes of the sulphate assimilation pathway and glutathione synthesis, including ATP sulphurylase, APS sulphotransferase, γ-glutamylcysteine synthetase and glutathione synthetase (Nussbaum et al. 1988, Ruegsegger et al. 1990, Ruegsegger and Brunold 1992). Presumably this also involved regulation of the sulphate transporters in response to the increased flux of S from sulphate into PCs.

In *Arabidopsis*, Cd and Cu, which promote the production of PCs, also promote expression of the mRNAs for γ-glutamylcysteine synthetase and glutathione synthetase (Xiang and Oliver 1998) implying control at the transcriptional level. Heavy metals that do not induce PC synthesis do not affect transcription of these genes. Supplying exogenous GSH or GSSG to the plants also did not promote transcription. Xiang and Oliver (1998) proposed that, in *Arabidopsis*, a signal transduction pathway that does not involve GSH or GSSG elicits the transcriptional response to heavy metals, perhaps involving jasmonic acid. Transcriptional regulation of the enzymes involved in the synthesis of glutathione from cysteine is consistent with the more sluggish restoration of the intracellular glutathione pool compared with the very rapid drop following the application of Cd which is thought to involve post-translational activation of PC synthase by Cd (Grill et al. 1989, Loeffler et al. 1989).

Some forms of environmental stress, unlike Cd and Cu, cause glutathione to accumulate in plant tissues (Table 1) implying that the rate of glutathione synthesis exceeds the rate of demand. In these instances, accumulation of glutathione appears to be an intrinsic part of the plant's response to stress and implies that the postulated regulatory mechanism involving inhibition of γ-glutamylcysteine synthetase by glutathione must be countermanded. Thus, Farago and Brunold (1990) demonstrated that two herbicide safeners enhanced accumulation of glutathione, sulphate assimilation, and cysteine and glutathione synthesis in maize roots. The safeners also increased the activities of both ATP sulphurylase and APS sulphotransferase. One of the safeners also enhanced sulphate uptake. Although these data were obtained 6 d after addition of the safeners, the increased rate of sulphate assimilation and the association of high enzyme activities with high glutathione concentrations is inconsistent with repression of sulphate assimilation by glutathione via the mechanisms shown in Figure 4. Moreover, the high glutathione levels found in the presence of the safeners indicate that the proposed feedback

inhibition of γ-glutamylcysteine synthetase by glutathione must have been overcome by another factor(s). One possibility is compartmentation: if glutathione is spatially separated from the site of γ-glutamylcysteine synthetase then feedback inhibition of this enzyme would be effectively eliminated. Also, the increased gene expression of γ-glutamylcysteine synthetase induced by the safeners could more than compensate for the post-translational inhibition by glutathione. Consistent with this, transformed poplar plants, which overexpress γ-glutamylcysteine synthetase activity, contain higher concentrations of glutathione.

CO-ORDINATION OF N AND S UPTAKE AND ASSIMILATION

In vegetative plants in which secondary compounds are not a significant sink for S, the molar N:S ratio is maintained at approximately 30 reflecting the N and S composition of protein. Therefore plants require N (acquired mainly as nitrate) and S (acquired mainly as sulphate) in a similar molar ratio. Regulation of sulphate and nitrate uptake must originate with a sensory mechanism in the root as this is the main site of uptake (Cram 1990). Nitrate assimilation occurs predominantly in the root of some species and in the shoot of others (Brunold 1993). Sulphate assimilation occurs mainly in the shoot in most plants (Brunold 1993).

Several processes are involved in coordinating nitrate and sulphate assimilation. The primary principle in all of these systems is that that low levels of the reduced form of one element (say S) has the effect of enhancing the assimilatory capacity for that element (S) and shutting down the assimilatory capacity of the pathway for the other element (N). Smith (1980) reported that transferring cultured tobacco cells to N-depleted medium decreased the activity of cysteine synthase but transferring the cells to S-depleted medium had no effect. This suggests that the activity of cysteine synthase is primarily regulated by the availability of N within the cells rather than by the availability of S (Smith 1980). Responses such as these include various regulatory mechanisms involving key cellular metabolites. For example OAS, a product formed from the non-S amino acid serine, appears to be very important in regulating aspects of sulphate assimilation. Plants also have mechanisms for increasing translational production of key enzymes involved in the two pathways. Here again, OAS appears to be important, enhancing sulphate assimilation by promoting production of the sulphate transporter. Other cellular and physiological processes appear to be important too. In plants in which nitrate assimilation in the root is significant (e.g. *Vicia*), this must involve inter-organ signalling between the root (where ni-

trate is assimilated) and the shoot (where sulphate is assimilated). In species in which both sulphate and nitrate assimilation occur mainly in the shoot, intracellular separation of specific events would appear to provide one way of effecting controlled coordination of the two processes. For example, assimilation of sulphate-S occurs exclusively in the chloroplast but nitrate assimilation involves both the cytosol (nitrate reduction) and the chloroplast (assimilation of nitrite into glutamate). Various scenarios are possible; for example the rate of nitrate reduction in the cytosol could control both cysteine and glutamate synthesis in the chloroplast or the demand for cysteine could control nitrate reduction in the cytosol, etc.

Consistent with the principles detailed above, some enzymes of N assimilation are down regulated by S deficiency. Various studies have reported decreased expression of nitrate reductase activity at low S (Pal et al. 1976, Friedrich and Schrader 1978, Reuveny et al. 1980, Haller et al. 1986). Friedrich and Schrader (1978) reported that glutamine synthetase activity is also decreased by S deprivation but to a lesser extent than nitrate reductase. Reuveny et al. (1980) showed that, in tobacco cell cultures, induction of nitrate reductase activity by nitrate was dependent on the initial supply of S even though the amount of soluble protein produced by the cells was largely independent of the amount of the sulphate supplied. This indicates that the S-dependent decrease of nitrate reductase activity was not a general consequence of decreased protein synthesis.

Experiments with cell cultures and intact plants in which both the level of N and S nutrition are varied provide further evidence for a link between the pathways of N and S assimilation. At high N but not at low N, ATP sulphurylase is de-repressed by S starvation (Reuveny et al. 1980, Barney and Bush 1985, Haller et al. 1986), indicating that cells regulate ATP sulphurylase activity according to their N status. In *Rosa* cells and in *Lemna minor*, low N nutrition restricts the activity of APS sulphotransferase, but has little effect on ATP sulphurylase (Brunold and Suter 1984, Haller et al. 1986). It appears that APS sulphotransferase is particularly significant for the coordination of N and S assimilation in at least some species (Haller et al. 1986, Neuenschwander et al. 1991).

Cysteine synthase activity is controlled by OAS, a product of N-assimilation. This metabolite is of interest for several reasons. Firstly, it serves as the sulphide acceptor for cysteine synthesis. OAS is synthesized from serine in a reaction catalysed by serine transacetylase:

$$\text{Serine} + \text{acetyl-CoA} \Rightarrow \text{OAS} + \text{CoA}$$

As noted above, the concentration of cysteine in cells is under tight control and this can only be achieved by regulating the availability of OAS and/or

sulphide. When OAS was fed to detached spinach leaves supplied with H_2S, the cysteine concentration increased indicating that OAS limited cysteine synthesis (Buwalda et al. 1992). Secondly, the flux of S from sulphate to cysteine appears to be influenced by OAS. When *Lemna minor* was kept in the dark for 24 h to establish negligible APS sulphotransferase activity, supplying OAS increased the APS sulphotransferase activity to 50 % of the activity of plants maintained in the light (Neuenschwander et al. 1991). This was associated with an increase in the total acid soluble thiol content, thereby establishing the importance of OAS in regulating sulphate assimilation. It is evident from the above that OAS is important in both regulating the rate of cysteine synthesis and in coordinating N and S assimilation. Cysteine itself could be involved in negative feedback regulation of OAS synthesis (Figure 4) as inhibition of serine transacetylase by cysteine has been demonstrated *in vitro* (Smith and Thompson 1971, Brunold and Suter 1982).

Other observations also suggest that OAS is important in linking N and S uptake and assimilation. Not only does OAS accumulate in S-stressed cells, conditions which promote S uptake, but it also accumulates in cells immediately after the alleviation of N stress (Smith 1980), consistent with enhanced S assimilation. Conversely, cells starved for N contain very low levels of OAS, conditions that would also shut down sulphate assimilation. Also, Hawkesford and Smith (1997) have reported that OAS induces uptake of sulphate and causes the accumulation of glutathione and cysteine in leaf tissues raising the possibility that OAS acts as a signal for the regulation of S uptake.

OAS is not the only metabolite that links S and N metabolism. Phosphohomoserine, formed through the action of homoserine kinase activity, links methionine biosynthesis to the mainstream synthesis of amino acids formed from aspartate (Bryan 1990). The possibility that phosphohomoserine might act in a similar way to OAS does not appear to have been investigated.

In *Capsicum annuum*, up-regulation of cysteine synthase increases cysteine synthesis (Romer et al. 1992) and this has been interpreted as implying that cysteine synthesis in this species is limited by cysteine synthase activity. However, it should be noted that most plants exhibit very high cysteine synthase activity. This suggests that the concentration of one of the substrates (OAS or sulphide, both of which normally occur at low concentration in plants) is also important, in much the same way that glutathione synthesis is probably more dependent on the availability of cysteine than upon the activity of γ-glutamylcysteine synthetase. Indeed, as noted above, the production of OAS in plants seems to be under tight control.

The uptake of nitrate in canola is decreased at low S (Lappartient and Touraine 1996), consistent with a mechanism for coordinating N and S assimilation when S is limiting. Presumably the nitrate uptake mechanism

responds to a signal that reflects the availability of a product of S assimilation.

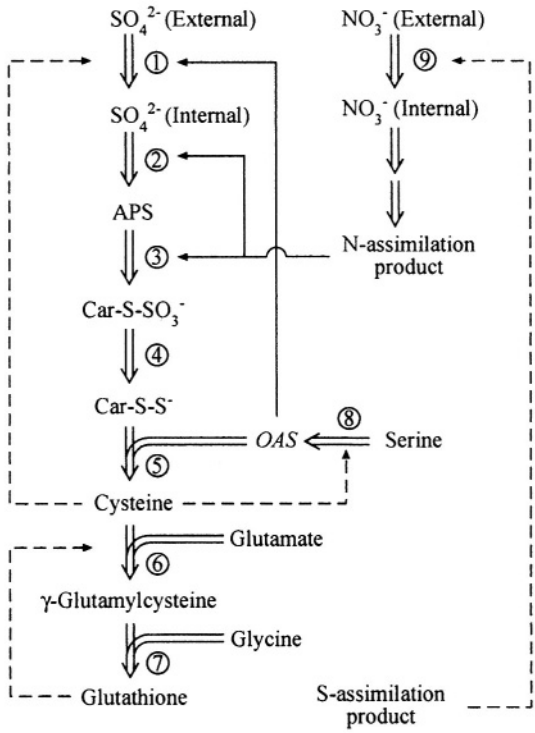

Figure 4. Summary of the regulation of the assimilation of sulphate-S into cysteine and glutathione and coordination with aspects of N assimilation. Enzyme-catalysed interconversions of metabolites are shown by double-tailed arrows. Regulatory effectors are shown with single tailed arrows. Those shown with discontinuous lines are inhibitory and those shown with continuous lines are stimulatory. Enzymes/proteins involved are: 1, sulphate membrane transporting protein(s); 2, ATP sulphurylase; 3, APS sulphotransferase; 4, organic thiosulphate reductase; 5, cysteine synthase; 6, γ-glutamylcysteine synthetase; 7, glutathione synthetase; 8, serine transacetylase; 9, nitrate uptake mechanism.

Remobilization of protein-S in vegetative plants appears to be determined primarily by N nutrition rather than S nutrition. While the availability of cysteine and OAS are probably important in regulating the assimilation of inorganic N and S, nothing is known about the signals for initiating protein hydrolysis and the production of glutathione from protein though the sensitivity of protein hydrolysis to N nutrition suggests that this probably involves a product of N metabolism rather than S metabolism.

LONG DISTANCE TRANSPORT OF S AND THE TRANSMISSION OF REGULATORY SIGNALS IN VEGETATIVE AND GENERATIVE PLANTS

Short-term fate of sulphate acquired by the root

Plants normally acquire their S as sulphate from soil via one or more high affinity sulphate transport proteins, which occur exclusively in root parenchyma. Very little of the sulphate acquired by root parenchyma cells is normally assimilated in the root (Cram 1983b). Thus, the metabolic activity of the root is not likely to be important in regulating sulphate uptake in these cells. In S-adequate plants, the sulphate acquired by root cells is both sequestered in root vacuoles and exported in the xylem but when plants are transferred to sulphate-free medium most of the label stored in root vacuoles is transferred into the xylem. Therefore the short-term distribution of sulphate is very dependent upon the S status of the plant. Collectively, these processes and the sites at which they occur have important implications for the control of sulphate assimilation in whole plants and provide important grounds for suspecting that glutathione, which is the major form of organic S in phloem (Rennenberg 1982, Lappartient and Touraine 1996), serves as the most important long-distance signalling metabolite for the control of sulphate uptake in the root.

The withdrawal of sulphate from root parenchyma of plants grown at very low S occurs passively in response to a decrease in the concentration of sulphate in the cytoplasm as sulphate is conducted from the root to the shoot in the xylem as a result of transpiration activity (Clarkson et al. 1993, Bell et al. 1995). If the activity of the sulphate uptake mechanism in root cells is controlled by the concentration of sulphate in root cytoplasm then the conduction of sulphate from root cytoplasm into the xylem would lead directly to enhanced sulphate uptake. However, there is no direct evidence linking the activity of sulphate uptake to the concentration of sulphate in the cytoplasm. Moreover, if such a system did exist, it would be unresponsive to the requirement for S at the sites of demand in the shoot.

The conduction of sulphate from root cytoplasm into the xylem is thought to be controlled by organic S. Herschbach and Rennenberg (1991) found that exogenous applications of glutathione and cysteine strongly inhibit movement of sulphate from root parenchyma into the xylem. This is consistent with the hypothesis proposed by Rennenberg and Lamoureux (1990) that glutathione acts as a signal for relaying the S status of the shoot to the root. In this way, glutathione would have two effects which could be mediated in

the root either directly or, since plants contain mechanisms for the interconversion of glutathione and cysteine, indirectly via cysteine. One effect concerns control of the high affinity sulphate transporter(s); the other concerns the loading of sulphate into the xylem. As argued before, there are theoretical reasons for suspecting that the glutathione response is mediated via cysteine since the cysteine pool is kept under tighter control and Rennenberg (1982, 1984) has proposed that glutathione can be considered as a reserve of reduced S.

Grain development in generative plants is associated with the production of storage proteins thereby necessitating the import of S by developing grains. In theory, S could be acquired directly from exogenous sources such as soil sulphate but this presupposes that generative plants have the capacity to acquire exogenous sulphate and conduct it in the transpiration stream, directly or indirectly, to developing grains. In cereals, the leaves are essentially fully expanded at this time (MacKown et al. 1992) so that there would be little competition for sulphate acquired in this way. However, direct utilization of exogenous sulphate for grain growth presupposes that developing grains possess mechanisms for the reductive assimilation of sulphate-S into the protein S-amino acids.

In practice, the acquisition of exogenous sources of inorganic nutrients, including sulphate, during generative growth is very dependent on water availability. Plants can acquire exogenous nutrients, including sulphate, under very moist or irrigated conditions (Smith and Lang 1988, Smith and Whitfield 1990, Larsson et al. 1991). Moreover, in generative wheat, the sulphate-S acquired in this way can be assimilated into grain storage proteins (Fitzgerald 1997). However, acquisition of exogenous S for grain growth is relatively unimportant in most non-irrigated regions of the world when drying soils at the time of generative growth restrict nutrient uptake (Gregory et al. 1979, Smith and Whitfield 1990). Thus, under these conditions, as discussed below, generative plants must acquire S for grain growth from the endogenous sources acquired during generative growth.

Short-term fate of sulphate arriving in the shoot from the root

In S-adequate vegetative plants, newly acquired S is delivered predominantly to the expanding leaves; fully mature leaves are quantitatively unimportant sinks for newly acquired sulphate (Smith and Lang 1988, Adiputra and Anderson 1992, Sunarpi and Anderson 1996). Since xylem transport directs movement of materials into leaves in proportion to their leaf area, this

implies that plants must have a mechanism for transferring sulphate from the xylem into the phloem so that it is delivered specifically to the expanding leaves (Smith and Lang 1988). Consistent with this, Smith and Lang (1988) found that when plants were treated with *m*-chlorophenylhydrazone, an inhibitor of phloem loading, sulphate was distributed to leaves in proportion to their leaf area. In stylo, the only sulphate transporter found in shoot material is SHST3, which has low affinity for sulphate.

Expanding leaves have an active sulphate assimilation pathway (Schmutz and Brunold 1982, Brunold 1993). For example, ATP sulphurylase is reportedly very active in expanding leaves but mature leaves exhibit very low activity (Adams and Rinne 1969). Much of the sulphate delivered to expanding leaves is assimilated into S-amino acids and incorporated into constitutive proteins as the leaf grows. Expanding leaves also contain high concentrations of glutathione although it is not clear how much of this is imported from other plant parts and how much is synthesized *de novo* from sulphate within the leaf.

In S-adequate plants, sulphate accumulates in leaf vacuoles. Since mature leaves exhibit very low net synthesis of protein and do not assimilate sulphate (Adams and Rinne 1969, Schmutz and Brunold 1982, von Arb and Brunold 1986, Brunold 1993), then leaf vacuolar sulphate is a potential reserve of sulphate which, in view of the slow rates of passive sulphate efflux across the tonoplast (Bell et al. 1994), can be slowly mobilized in the event of a decrease in the sulphate concentration in the cytoplasm (e.g. S stress). Consistent with this, the mature leaves of plants which have been grown under S-limiting conditions do not synthesize additional protein when the plants are provided with adequate S although the sulphate content of the leaves might rise considerably (Smith and Lang 1988, Dietz 1989). Sulphate-S in leaf vacuoles effluxes slowly in vegetative plants. Consistent with this, pulse chase experiments with S-adequate plants indicate that a large proportion of the S acquired by a leaf while it is expanding is not incorporated into the insoluble (protein) fraction and is subsequently exported from the soluble fraction over a very extended period of time (Adiputra and Anderson 1992). The rate of export of S from leaves of vegetative plants does not appear to be especially responsive to the imposition of S deficiency (Adiputra and Anderson 1995, Sunarpi and Anderson 1996) but this is not the case in generative plants (Fitzgerald et al. 1999a) where developing grains impose a very strong demand for S.

The discussion above has focussed on the redistribution of S that is delivered in the xylem to mature leaves. However, it should be noted that redistribution of S from the leaves of vegetative cereals and soybean is evident well before they attain full expansion (Adiputra and Anderson 1992, Sunarpi and Anderson 1996). Given that expanding leaves actively assimilate sulphate

into cysteine and glutathione then the possibility that a significant amount of S found in the phloem originates from these leaves as glutathione rather than as sulphate (as is the case in mature leaves) needs to be addressed.

Transport within the shoot and recycling via the root

Since expanding leaves are the principal site for the assimilation of sulphate into cysteine and glutathione (Brunold 1993) and roots are not quantitatively active in this way (Cram 1983b), then leaves must be an important source for the long distance transport of organic S to sites that do not actively assimilate sulphate, both within the shoot itself and to the root. This must involve the long distance transport of glutathione since the phloem must act as the conduit for the transport of organic S from the shoot to the root and glutathione is the major form of organic S in phloem (Lappartient and Touraine 1996). This has several implications. Most importantly, it provides a mechanism for conveying a signal about the S status of the shoot to the root which can be used to regulate the activity of the sulphate uptake mechanism and also influence the loading of sulphate from the cytoplasm of root parenchyma into the xylem and hence movement of sulphate from root vacuoles via the cytoplasm into the xylem as proposed by Rennenberg et al. (1989). Second, it implies a phloem unloading mechanism in the root and raises the question of the eventual fate of the glutathione in the root. Presumably it can serve as a source of reduced S for the formation of essential S-compounds (e.g. cysteine and methionine for incorporation into proteins) required for root growth and maintenance. Another possibility is that glutathione is recycled to the shoot in the transpiration stream where it is perhaps reloaded into the phloem a second time (S recycling hypothesis), a process that would involve phloem to xylem transfer in the root.

Evidence for S recycling of endogenous S (Figure 5) has been obtained using a split-root technique (Cooper and Clarkson 1989, Larsson et al. 1991). In wheat, S recycles as sulphate and recycling S does not mix with other S pools within the root (Larsson et al. 1991). However, definitive demonstrations that endogenous glutathione, emanating from the shoot, also recycles still provides a formidable technical challenge.

Since sulphate recycles, this raises the question whether sulphate arriving in the root in the phloem could act as a signal for regulating sulphate uptake in the root. To date, there is no experimental evidence for excluding this possibility.

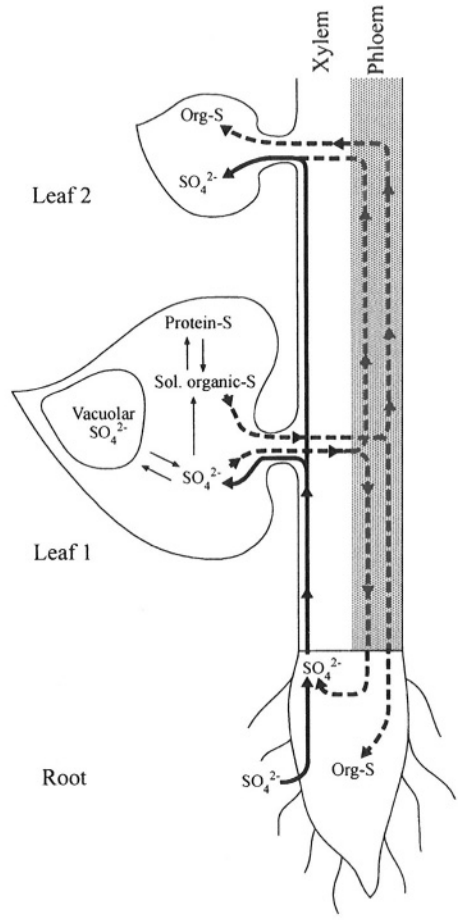

Figure 5. Representation of long distance S transport in the xylem and in the phloem in a vegetative plant in which leaf 1 denotes a mature fully-expanded leaf and leaf 2 a leaf in the early stages of expansion.

In generative plants grown with adequate water, exogenous sulphate is the main form of S for the synthesis of grain proteins (Fitzgerald et al. 1999b). However, under normal field conditions, without addition of abundant water, the main endogenous sources of soluble S which are used for grain growth are sulphate and glutathione acquired/formed in vegetative tissues from S during vegetative growth. Sulphate occurs in the vacuoles of roots, leaves and stems and diffuses passively into the cytoplasm when the cytoplasmic concentration falls below a critical concentration (Clarkson et al. 1983). To reach the grain, sulphate has to be loaded into the phloem since

redistribution via the xylem would direct sulphate to transpiring tissues. In any event, the apoplastic discontinuity to the endosperm cavity (Zee and O'Brien 1970) from which metabolites are recruited into the developing endosperm (Ugalde and Jenner 1990a) would prevent direct delivery of sulphate via the xylem. As discussed below, developing grains appear to have the capacity to incorporate sulphate-S into protein-S so therefore they must have mechanisms for recruiting sulphate from the endosperm cavity. Sulphate occurs in the developing grains of wheat plants grown under S-sufficient conditions (Roberts and Koehler 1966) and, under these conditions, is a quantitatively important source of S for grain development (Fitzgerald 1997). This is consistent with earlier observations which indicate that the amount of S delivered as cysteine and methionine is far too small to account for the S occurring in the S-amino acids in grain proteins (Fisher and MacNicol 1986, Blumenthal et al. 1990, Ugalde and Jenner 1990b). However, in plants grown with inadequate S, the amount of endogenous sulphate-S available for redistribution to developing grains is negligible (Fitzgerald et al. 1999b) and under these conditions, the S required for grain growth must come from other endogenous sources.

Glutathione is a quantitatively important form in which S is imported into developing grains (Fitzgerald 1997). Indeed, in plants grown at low S during vegetative growth, glutathione accounts for about 86 % of the soluble S in the endosperm cavity suggesting that it is the main form in which S is transported in these plants. The transported glutathione is probably derived from at least two sources. One probably concerns the free glutathione present as a constitutive metabolite in chloroplasts. Presumably glutathione is recruited from this pool during generative growth, perhaps by some form of regulated senescence or enhanced membrane permeability to glutathione. The other likely source is protein-S.

Role of glutathione in the remobilization of protein-S in vegetative plants

Remobilization of protein-S can be readily demonstrated in germinating seeds. In soybean, for example, the amount of insoluble S in the cotyledons declines to very low levels as germination proceeds (Sunarpi and Anderson 1995). This is accompanied by a transitory rise in the concentration of soluble S in the cotyledons. Subsequently, the S lost from the insoluble fraction in the cotyledon is quantitatively recovered in the growing seedling (Sunarpi and Anderson 1995). Similarly, in germinating barley, the amount of insoluble S in the endosperm declines during germination and the amount of glu-

tathione and soluble (free) methionine increases sharply (Imsic and Anderson, unpublished data). The production of glutathione implies that germinating barley has a mechanism for mobilising S from storage proteins into cysteine and/or methionine (or products derived from them) and incorporating the S into the cysteinyl residues of glutathione. Presumably this involves processes similar to those depicted in Figure 2. This raises interesting questions about the control of glutathione synthesis during seed germination and its coordination with protein hydrolysis in germinating seeds.

N stress promotes a marked increase in the export of protein-N from mature leaves to developing leaves (Mei and Thimann 1984). Conversely, mobilization of protein-S from mature leaves of vegetative soybean plants grown at high N is not especially responsive to S deficiency (Sunarpi and Anderson 1996). However, the loss of insoluble S (protein-S) from mature leaves of vegetative soybean grown at low S is very sensitive to N stress (Sunarpi and Anderson 1997b). The loss of insoluble S is accompanied by a slightly greater proportional loss of insoluble N implying the preferential hydrolysis of S-poor proteins from mature leaves. These data are not inconsistent with the hydrolysis of vegetative storage proteins; these proteins, which are known to occur in soybean leaves, are relatively poor in S amino acids, and are hydrolysed in response to low N (Staswick 1994). The loss of protein S from mature leaves of N-stressed soybean plants is accompanied by a minor increase in the concentration of glutathione in these leaves and a very large increase in glutathione in the young developing leaves at the shoot apex (Sunarpi and Anderson 1997b). These data are consistent with N-stress induced hydrolysis of protein-S and synthesis of glutathione in the mature leaves linked to transport of glutathione to the young leaves at the shoot apex where it accumulates, presumably because the level of N nutrition is too low to support the synthesis of constitutive proteins required for new growth. Perhaps this would prove a suitable system for examining whether glutathione provides the signal for arresting sulphate uptake in the root.

Role of glutathione in the remobilization of protein-S to developing grains

It is well known that the level of leaf protein falls during grain growth, especially in N-stressed plants (Neales et al. 1963, Dalling et al. 1976, Dalling and Simpson 1981, Simpson et al. 1983, MacKown et al. 1992). Largely for this reason, protein-S has long been thought to be an important precursor of the S imported into developing grains since hydrolysis of leaf protein would be expected to release soluble forms of S as well as N. Recently, this

hypothesis has been examined by several groups although the effect of N nutrition on the mobilization of leaf protein-S for grain growth has yet to be examined. Generative cereals exhibit strong mobilization of protein-S in response to S stress (Fitzgerald et al. 1999a,b). Indeed, in plants grown at low S during vegetative growth the loss of insoluble S from vegetative tissues quantitatively accounts for the gain of protein-S in the developing grains. Fitzgerald (1997) examined the S composition and turnover of the contents of the rachis, endosperm cavity, and endosperm and calculated that glutathione accounted for about 84 % of the S imported into developing grains in plants supplied with inadequate S during vegetative growth. The corresponding figure for plants grown with adequate S during vegetative growth was 37 %, most of the balance being supplied by sulphate. Since leaf protein-S accounted for all of the S imported into the grains in the plants grown with inadequate S, this implies that almost all of the remobilized leaf protein-S was metabolized to glutathione, again emphasising the importance of processes such as those shown in Figure 2.

The above data draw attention to the importance of glutathione synthesis in senescing leaves and cotyledons as a way of mobilising S from proteins. Little is known about the types of proteins from which S is recruited and this draws attention to the fact that leaves and the storage tissues of seeds contain specific storage proteins which differ greatly in their S content as shown for the storage proteins of wheat endosperm (Table 2). In addition to the vegetative storage proteins described above, leaves contain very large amounts of the functional protein ribulose bisphosphate carboxylase, which is also subject to proteolysis (Vierstra 1993). The mobilization of S from proteins such as these and the pathway for the inferred incorporation of methionine-S into glutathione and its regulation remain as challenges for the future. Another matter still to be addressed is the mechanism of glutathione uptake from the endosperm cavity and its presumed metabolism to cysteine and methionine in developing grains.

Table 2. Cysteine (Cys) and methionine (Met) contents of some major wheat storage proteins.

Protein	No. of amino acid residues			Composition	
	Total	Cys	Met	%N	%S
HMW Glutenin[1]	842	4	3	18.3	0.61
LMW Glutenin[2]	354	8	6	18.9	1.10
α/β-Gliadin[3]	243	5	2	17.9	0.87
γ-Gliadin[4]	292	9	6	17.3	1.45
ω-Gliadin[5]	330	0	0	18.5	0.00

Sources: 1, Sugiyama et al. 1985; 2, Pitts et al. 1988; 3, Garcia-Maroto et al. 1990; 4, Rafalski 1986; 5, Castle and Randall 1987.

ROLE OF GLUTATHIONE IN THE ASSIMILATION OF INORGANIC SELENIUM

Selenoproteins and selenium toxicity

Selenium (Se) is the higher homologue of S and it shares many common features with S (Anderson and Scarf 1983). In animals and some bacteria, trace amounts of Se are known to be essential for growth and maintenance. In geographic regions that have an inherently low Se level in the soil, Se deficiency is well recognized in domestic animals, especially sheep, and action has to be taken to ensure that stock receive an adequate amount of Se. The biological activity of several proteins in animals is dependent on the presence of Se. These proteins, known as selenoproteins, contain Se in specific selenocysteinyl residues in which the S atom of cysteine contains Se in place of S. The synthesis of selenoproteins must involve Se-specific mechanisms which distinguish between Se and S. The codon and corresponding tRNA for selenocysteine have now been identified, causing selenocysteine to be referred to as the '21st amino acid' (Bock et al. 1991a,b). It is common for selenoproteins to contain cysteinyl residues in addition to selenocysteine. For example, each of the four sub-units of glutathione peroxidase from erythrocytes contains one residue of selenocysteine and two cysteinyl residues (see Anderson 1993). At present, there is no definitive evidence for the existence of selenoproteins in higher plants (Anderson 1993, Lauchli 1993) although a gene sequence from plants with homology to the Se-dependent glutathione peroxidase in animals has been reported (Criqui et al. 1992). Similarly an absolute requirement for Se has not been demonstrated in plants (Anderson 1993, Lauchli 1993) although the growth of some algae appears to either require Se or is stimulated by Se (Price et al. 1987).

Although it is unclear whether higher plants contain selenoproteins, the mechanism for the specific incorporation of selenocysteine residues at specific sites during peptide formation is now well understood in other organisms (Bock et al. 1991a,b). This involves the aminoacylation of a specific serine-accepting tRNA ($tRNA^{ser}$) to form $seryl\text{-}tRNA^{ser}$. The attached seryl moiety is then phosphorylated ($phophoseryl\text{-}tRNA^{ser}$) and this complex then reacts with a selenol to form $selenocysteinyl\text{-}tRNA^{ser}$:

$$Phosphoseryl\text{-}tRNA^{ser} + R\text{-}SeH \Rightarrow selenocysteinyl\text{-}tRNA^{ser} + R + Pi$$

The $selenocysteinyl\text{-}tRNA^{ser}$ is recognized (and also distinguished from $seryl\text{-}tRNA^{ser}$) by a specific code for selenocysteine in the mRNA for the protein being synthesized (Bock et al. 1991a,b). In this way the selenocys-

teinyl residue is incorporated into a specific position as the selenoprotein is formed. If plants have a similar mechanism, then attention will turn to the origin and nature of the compound R-SeH in the equation given above. The mechanism for the synthesis of GSSeH given in Figure 6 provides a potential explanation. In this event, glutathione would play an important role in the synthesis of selenoproteins in plants.

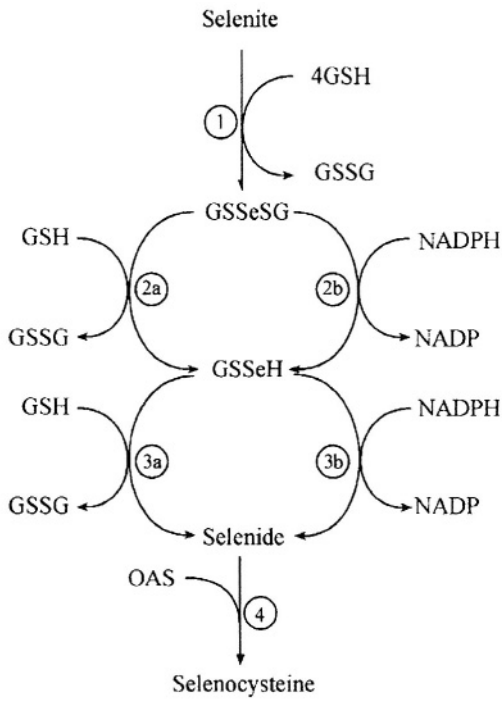

Figure 6. Postulated pathways for the reductive assimilation of inorganic selenite into selenocysteine in plants showing the involvement of glutathione. Reactions 1, 2a and 3a are non-enzymic. Reactions 2b and 3b are catalysed by glutathione reductase and reaction 4 by cysteine synthase.

Although Se is essential for some organisms, it is more generally regarded as a toxic element, largely because organisms, particularly plants, incorporate Se non-specifically in place of S into various key S-containing metabolites (Shrift 1969, 1973, Anderson and Scarf 1983). This can be likened to the replacement of phosphorus with arsenic in various metabolites during arsenic toxicity, thereby impairing the biological activity of the substituted metabolite. Proteins containing non-specific replacement of cysteine

and methionine residues by the respective Se-isologues, by definition, are not selenoproteins. Since the catalytic activity of many enzymes is determined by the -SH group of cysteinyl residues, it follows that non-specific replacement of S by Se in these proteins profoundly influences their catalytic activity. Selenoproteins by contrast require Se for protein function (Anderson 1993).

Availability and uptake of inorganic selenium compounds by plants

The most stable form of S in most aerobic soils is sulphate (oxidation state -6). In general, sulphite (oxidation state -4) is oxidized chemically and biologically to sulphate. However, the most stable form of Se in most aerobic soils (which tend to be acidic) is selenite (-4) rather than selenate (-6) although increasing soil alkalinity results in a shift to selenate (Lauchli 1993).

It is unclear whether selenate or selenite is the predominant form of Se taken up by plant roots under field conditions. Since selenite is the most abundant form of Se, the view has been expressed that in most aerobic soils, selenite is the form most readily available to plants (Anderson and Scarf 1983). However, selenite is tightly bound by soil components (Lauchli 1993). Consistent with this, Mikkelsen et al. (1988) have reported that plants take up selenate more readily than selenite.

Selenate acts as a very effective isologue of sulphate in several systems in plants. In particular, the sulphate uptake mechanism of plant roots supports the uptake of selenate. Thus, selenate and sulphate compete for uptake (Leggett and Epstein 1956, Smith 1976) and for binding sites on the sulphate transporter protein (Breton and Surdin-Kerjan 1977). The uptake competition between sulphate and selenate, and more generally between S and Se, has flow-on effects with respect to the S and Se content of tissues (e.g. Barak and Goldman 1997, Kopsell and Randle 1997) and the production of specific metabolites (e.g. Nigam and McConnell 1973). In general, S decreases Se uptake and vice versa. Similarly, S decreases the incorporation of Se into Se isologues of S-containing compounds and vice versa. Consistent with this, selenate, inhibits the incorporation of sulphate-S into glutathione (De Kok and Kuiper 1986).

Selenite is apparently taken up by a separate mechanism to selenate, perhaps passively, as selenite does not accumulate in the root against a concentration gradient (Lauchli 1993). Whilst plants readily metabolize selenite to selenocysteine (see below), it appears that plants can also oxidize selenite.

Asher et al. (1977) reported that the xylem of tomato plants grown in the presence of selenite, contained selenate, but not selenite.

Role of glutathione in assimilatory reduction of selenite and selenate into selenoaminoacids

Plants have very active mechanisms for the assimilation of inorganic Se (Brown and Shrift 1982, Anderson and Scarf 1983, Anderson 1993). These mechanisms are partly linked to and partly independent of the sulphate assimilation pathway. However, the roles of glutathione in the assimilation of inorganic S and inorganic Se are very different. In S assimilation, glutathione is an end-product and acts as a regulator of sulphate assimilation but in Se assimilation, glutathione acts directly as the reducing agent for the formation of selenide which is then incorpoorated into selenocysteine (Figure 6).

Selenate acts as a very effective analogue of sulphate for the enzyme ATP sulphurylase which catalyses the reaction:

$$\text{ATP} + \text{sulphate} \Rightarrow \text{APS} + \text{PPi}$$

In fact, the enzyme exhibits higher affinity for selenate than for sulphate (Shaw and Anderson 1972). Indeed, the decreased concentration of glutathione observed in selenate-treated plant tissues has been attributed to inhibition of sulphate metabolism caused by sulphate/selenate competition for the sulphate binding site on ATP sulphurylase (De Kok and Kuiper 1986, Bosma et al. 1991). With selenate as a substrate, the enzyme catalyses the formation of the Se analogue of adenosylphophosulphate (APS), APSe (Dilworth and Bandurski 1977). APSe is highly unstable but, in the presence of glutathione, the selenium-containing moiety can be readily reduced to elemental selenium (Dilworth and Bandurski 1977). The most likely route for the non-specific entry of Se into metabolism in most plants is thought to involve the sequence shown in Figure 6. It entails the reduction of selenite (acquired either directly, or indirectly from selenate via ATP sulphurylase activity) by glutathione (or by glutathione plus NADPH) to selenide which is then incorporated into OAS to form selenocysteine (Ng and Anderson 1978). The postulated role of glutathione in the assimilation of inorganic Se (Figure 6) would have the effect of exacerbating S/Se antagonisms, since the assimilation of Se would place an extra demand on the consumption of glutathione.

The initial reaction shown in Figure 6 for the reduction of selenite involves a non-enzymic reaction with glutathione as reductant. In theory this

could involve a thiol other than glutathione. However, glutathione is by far the most abundant thiol in most plants and it is the only thiol for which there is an active mechanism for regenerating the reduced form from the disulphide. The latter process involves the enzyme glutathione reductase, which is extremely active in chloroplasts. Since the reaction catalysed by this enzyme uses NADPH which in turn is formed via the light reactions, then illuminated chloroplasts can reduce GSSG with the evolution of O_2 (Jablonski and Anderson 1978):

$$H_2O + NADP^+ \Rightarrow 0.5O_2 + NADPH + H^+$$

$$NADPH + H^+ + GSSG \Rightarrow 2GSH + NADP^+$$

Given this, it follows from Figure 6 that illuminated chloroplasts have the potential to support the reduction of selenite (and GSSeSG) with the production of O_2 (i.e. selenite-dependent O_2 evolution). Consistent with this, illuminated chloroplasts reduce selenite (and GSSeSG) at extremely rapid rates (Jablonski and Anderson 1982), far in excess of the rates needed to account for the reductive assimilation of Se in whole plants.

Except for the reaction involving ATP sulphurylase acting on selenate, the reactions involved in the formation of selenide do not directly involve enzyme-catalysed reactions. However, plants, especially Se-accumulator plants, produce a wide range of Se isologues of various S-containing metabolites including selenomethionine, selenocystathionine and many others (Shrift 1969, 1973). The formation of these compounds would only be possible if the enzymes, which normally act on S-containing substrates also support the non-specific formation of the corresponding Se isologues. The most important reaction of this type is the formation of selenocysteine from OAS and selenide (in place of sulphide) catalysed by cysteine synthase (Ng and Anderson 1978) since, in theory, selenocysteine can serve as the substrate for the formation of the various other Se isologues of S substrates found in plants exposed to Se. Enzymes in addition to cysteine synthase which are known to support non-specific reactions with Se include cystathionine γ-synthase and β-cystathionase (McCluskey et al. 1986, Dawson and Anderson 1988), the latter two reactions accounting for the formation of selenocystathionine and homoselenocysteine, respectively. Selenomethionine is also synthesized by plants exposed to Se and incorporated, non-specifically, into protein (Eustice et al. 1980). These observations imply that the binding sites of the enzymes involved cannot distinguish between the normal S-containing substrate and the corresponding Se isologue.

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Chapter 5

THE ROLE OF GLUTATHIONE IN PLANT RESPONSE AND ADAPTATION TO NATURAL STRESS

Michael Tausz

INTRODUCTION

The tripeptide glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is the main low molecular weight thiol in most plant tissues. Due to the particular properties of the molecule it plays multiple roles in cellular metabolism. It is a central compound in sulphur metabolism and is considered the main transport form of reduced sulphur (Rennenberg and Lamoureux 1990). It links the sulphur reduction pathways to the protein synthesis and functions as a buffer for reduced sulphur. Glutathione also plays an important role in the scavenging of toxic reactive oxygen species (ROS). The activation of dioxygen (O_2) is an important aspect of the cell metabolism (Elstner 1982). The active oxygen species formed in the course of oxygen activation processes are highly reactive and difficult to keep under control. The action of ROS in cells is called oxidative stress. Oxidative stress is an inescapable feature of life in an oxygen atmosphere and ROS are involved in nearly all effects of environmental stresses on plants (Elstner and Osswald 1994, De Kok and Stulen 1993). The capacity of the glutathione redox system to detoxify dangerous ROS is potentially dependent on the pool size of total GSH, on the redox ratio GSH/GSSG (GSSG = oxidized glutathione), and on the activity of the regenerating enzyme system, the NADPH-dependent glutathione reductase. Elevated levels of GSH appear to be correlated to active plant responses to environmental stress and responses of GSH synthesis, GSH redox status, and GSH related enzyme activities have been found repeatedly in plants under stress (Alscher 1989).

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METABOLISM IN PLANT CELLS UNDER STRESS – REACTIVE OXYGEN SPECIES (ROS)

Molecular oxygen (O_2, dioxygen) has the unusual chemical property that it has two unpaired electrons with parallel spins. The ground-state oxygen molecule is triplet and excitation by energy transfer leads to highly reactive singlet state oxygen (singlet oxygen). The particular chemistry of oxygen also means that an univalent reduction (by accepting one single electron) is probable. This reaction yields oxygen free radicals. Free radicals are molecules with one unpaired electron, many of them very reactive. In the redox interconversion chain from O_2 to H_2O which requires a total of 4 electrons per O_2 molecule several intermediates (ROS) exist. All of them are less stable than O_2 or water. Reactive oxygen species include the superoxide anion free radical ($O_2^{-\bullet}$), the hydroxyl free radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) or the singlet oxygen. Oxidative attack initiates lipid peroxidation, protein oxidation, DNA damages, and pigment bleaching (Elstner and Oßwald 1994).

An increased production of reactive oxygen species is typical for the effect of most environmental stresses on plant cells. Biochemically, stress situations can be characterized as a transition from mainly two-electron (ionic) reactions to one-electron reactions (radical reactions) within the cells (Elstner and Oßwald 1994). Whereas the ionic reactions are tightly controlled, radical reactions easily escape metabolic controls and proliferate in chain reaction pathways.

In plant cells, the most important locations of ROS production are the chloroplasts (for a detailed review see Foyer and Noctor 2000). The most common mechanism of stress induced ROS production in the chloroplasts is based on an imbalance between the consumption of reductant (NADPH) in carbon fixation, and the need of the electron transport chain for the regenerated electron acceptor at the PS I site (NADP). Figure 1 illustrates the formation of ROS in plant chloroplasts under stress. Most environmental stresses impair the function of the Calvin cycle (i.e., the CO_2 fixation and the NADPH and ATP consumption). At the same time, light driven electron transport remains fully active, which leads to an overreduction of the electron transport chains and forces electrons to leak to alternate acceptors, predominantly to molecular oxygen (O_2). The univalent reduction of O_2 by leaking electrons yields the superoxide anion $O_2^{-\bullet}$ (Figure 1). Superoxide is a free radical with potentially destructive effects on proteins, lipids, nucleic acids, and pigments. For example, it may initiate free radical chain reactions in membrane lipids, which produce lipid peroxides and lipid hydroperoxides. Superoxide is detoxified enzymatically by superoxide dismutase (SOD). This reaction forms H_2O_2 which is enzymatically detoxified by the activities

of peroxidases. Peroxidases use another substrate as a reductant. Alternatively, H_2O_2 is also detoxified by catalase which catalyses the disproportion of H_2O_2 to the products O_2 and H_2O. A direct transfer of excess excitation energy from the photosystems to molecular oxygen yields the singlet oxygen, which is highly reactive. Free radical chain reactions may also yield the hydroxyl free radical ($OH^{\bullet}$), the most reactive of all these molecules. Unlike the superoxide and hydroxyl radicals, hydrogen peroxide and singlet oxygen are not free radicals, but nevertheless highly reactive oxygen species that are potentially dangerous to cell metabolism.

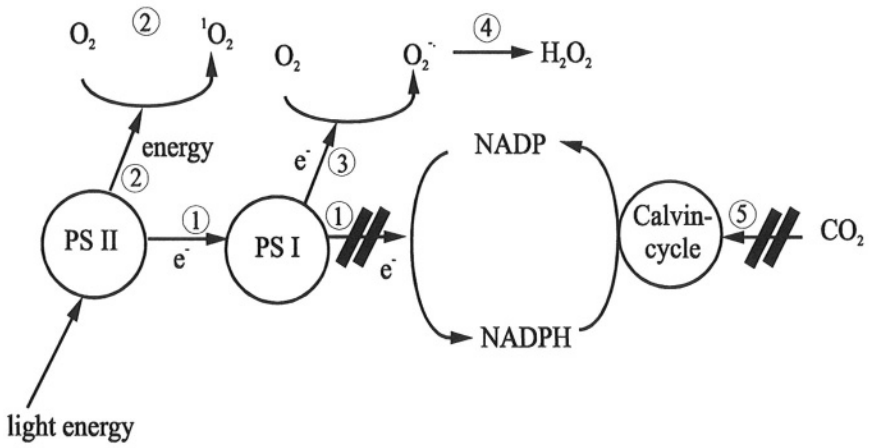

Figure 1. Some possible mechanisms of ROS formation in plant chloroplasts under stress: Every stress impact that causes a slower function or malfunction of the Calvin cycle (5) leads to the exhaustion of the primary electron acceptor NADP and to a block in the electron transport to NADP (1). Electrons leak to oxygen (3) yielding superoxide and, via superoxide dismutase reaction (4), H_2O_2. Excess excitation energy may be directly transferred to molecular oxygen yielding singlet oxygen (2). PS = Photosystem.

The chloroplast is the most important, but not the only site of stress related ROS production. The electron transport chains in the mitochondria are also a potential source for side-reactions producing ROS. Although the total rates of the mitochondrial ROS production are considered to be much lower than in the chloroplast, values are rarely cited (Foyer and Noctor 2000).

Limiting CO_2 is the direct consequence of various environmental stress impacts (Figure 1). For example, limited water supply leads to stomatal closure and CO_2 shortage in the assimilating mesophyll tissues. Under these conditions, an important adaptation of the photosynthetic system is photorespiration (for a detailed discussion see Asada 1999). In the course of this reaction sequence, H_2O_2 is produced in peroxisomes where it is scavenged by high activities of catalase. Certain stress situations or deficits in the cata-

lase systems can lead to oxidative stress caused by photorespiratory H_2O_2. Furthermore, photorespiratory activity also yields glycine, which is in turn an important reactant in GSH biosynthesis.

With increasing stress, cell metabolism is severely disrupted. When membranes and cell compartments are destroyed, the chain reactions described above occur at membrane lipids and proteins, and can further destroy cellular organization. In the course of such destructive reaction the formation of free radicals is augmented in all cellular compartments (Elstner and Oßwald 1994).

THE ROLE OF GSH IN THE ANTIOXIDATIVE DEFENCE SYSTEM OF PLANT CELLS

Since a certain amount of ROS is produced under normal metabolic conditions (Foyer and Noctor 2000), plants have developed effective defence systems to survive. These systems include a number of different functions, such as protection against excess energy absorption, alternative regeneration of the electron acceptor (NADP), the scavenging of ROS, the repair of oxidatively damaged structures, and regulation processes.

Glutathione may participate in different parts of the defence systems:

(1) Due to its sulphydryl group, the GSH molecule has strong antioxidative properties. It may react directly with ROS or with oxidized substrates reducing them. By doing so GSH is eventually transferred to its oxidized form, GSSG (Figure 2). Compared to another important cellular antioxidant, ascorbate, the rate constants of the direct reaction of GSH with superoxide and singlet oxygen are of comparable magnitude, but ascorbate concentrations in the chloroplast as well as average tissue concentrations of ascorbate appear to be 10- to 50-fold higher than those of GSH (Table 1). Since the antioxidative properties *in vivo* are dependent on both the rate constants and the concentrations in situ, the importance of GSH as a chemical scavenger of ROS is supposed be rather low (Rennenberg and Brunold 1994).

Table 1. Rate constants and concentrations of water soluble cellular antioxidants (composed after Polle and Rennenberg 1994, Polle 1997).

Antioxidant	Estimated concentrations in chloroplasts [mmol L^{-1}]	Oxidant	Rate constant K [L mol^{-1} s^{-1}]
Glutathione	0.2 - 2.4	Singlet oxygen	$2.4 * 10^6$
		$O_2^{-\bullet}$	$7 * 10^7$
Ascorbate	(2) 12 - 25	Singlet oxygen	$1 * 10^7$
		$O_2^{-\bullet}$	$2 * 10^7$

(2) GSH is required for the regeneration of another major cellular antioxidant, ascorbate. In that reaction, GSH is oxidized to GSSG and regenerated by glutathione reductase (GR, EC 1.6.4.2) using the reducing power from the photosynthetic electron transport chain (NADPH). This enzymatic pathway, often called Asada- or Foyer-Halliwell-cycle, is supposed to be the primary way of removing hydrogen peroxide and simultaneously regenerating the electron acceptor NADP. The capacity of this cycle may depend upon the activities of the enzymes and the concentration of the antioxidants (Figure 2). Changes in the turnover rates may become manifest in changes of the redox ratios dehydroascorbate/ascorbate or GSH/GSSG.

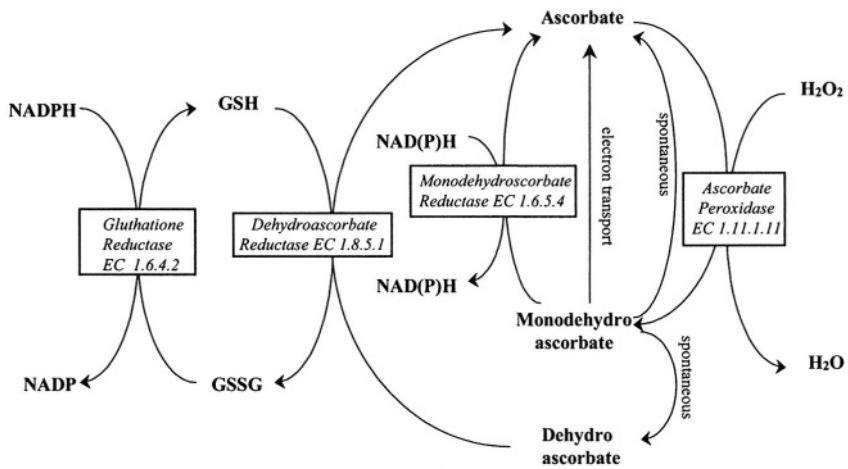

Figure 2. The glutathione-ascorbate cycle. GSH glutathione, GSSG oxidized glutathione, AS ascorbate, DHAS dehydroascorbate, MDAS monodehydroascorbate. AS is oxidized in a multitude of reactions to DHAS (not shown) or serves as a substrate for asorbate peroxidase. The predominant product of this reaction is MDHAS, which disproportionates spontaneously into AS and DHAS.

(3) Due to its antioxidative properties GSH may stabilize membrane structures. Membrane lipids are a prominent target for ROS reactions and lipid peroxidation forms acyl peroxides, which can be removed by GSH (Price et al. 1990 in McKersie and Leshem 1994). However, the most important compound playing this role in plant cells is the lipophilic antioxidant α-tocopherol, which is regenerated by ascorbate (Fryer 1992).

(4) GSH may reduce disulphide bonds of proteins and thus counteract the detrimental effects of ROS because the formation of protein disulphides is one of the consequences of oxidative attack. Furthermore, the thiol-disulphide exchange is of more general importance in enzyme activations, protein protection, and cellular regulation processes (Kunert and Foyer 1993).

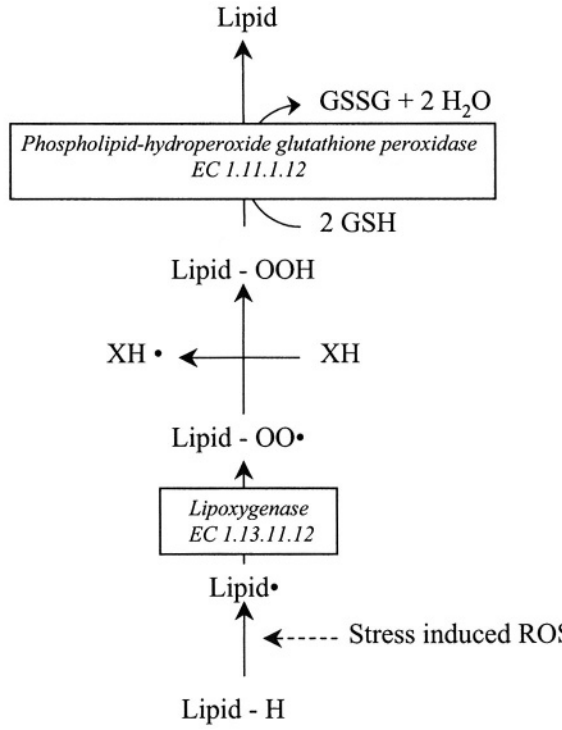

Figure 3. Proposed role for the plant phospholipid hydroperoxide glutathione peroxidase in the removal of toxic lipid peroxidation products (after Eshdat et al. 1997).

(5) A non-enzymatic reduction of dehydroascorbate by GSH is possible at a pH greater than 7 and GSH concentrations above 1 mmol L^{-1}. Within the living plant cell such a milieu exists in the stroma of illuminated chloroplasts with a pH about 8 and sufficient GSH concentrations (McKersie and Leshem 1994, see Table 1). The importance of this reaction *in vivo* is still not clear.

(6) GSH may serve as a substrate for peroxidase reactions (Figure 3). In animals, a selenium containing glutathione peroxidase (GPX, EC 1.11.1.9) has been demonstrated. In plant systems, increased GPX activities were measured upon stress impacts, but some of the reported activities may be due

to side reactions of glutathione *S*-transferases (GST, EC 2.5.1.18, see below). Only recently, a protein of strong homology to animal GPX (and distinctly different from GSTs) was characterized unequivocally in *Citrus sinensis*. The best substrate for this enzyme is not H_2O_2, but phospholipid hydroperoxides, which are toxic products in ROS-induced lipid peroxidation reaction chains. That means that the *Citrus* enzyme is a phospholipid-hydroperoxide glutathione peroxidase (EC 1.11.1.12) rather than a GPX. A role in removing toxic products of oxidative stress was ascribed to this enzyme (Figure 3). In contrast to the animal enzyme, the plant GPX has a cysteine in the active centre instead of a Se-cysteine and the specific activity of the plant enzyme is much lower (Eshdat et al. 1997). In pea and *Arabidopsis* it was shown that genes for a chloroplast targeted GPX exist and respond to stress (Mullineaux et al. 1998). However, the specific functions and the substrate of this enzyme *in vivo* are still hypothetical (Eshdat et al. 1997).

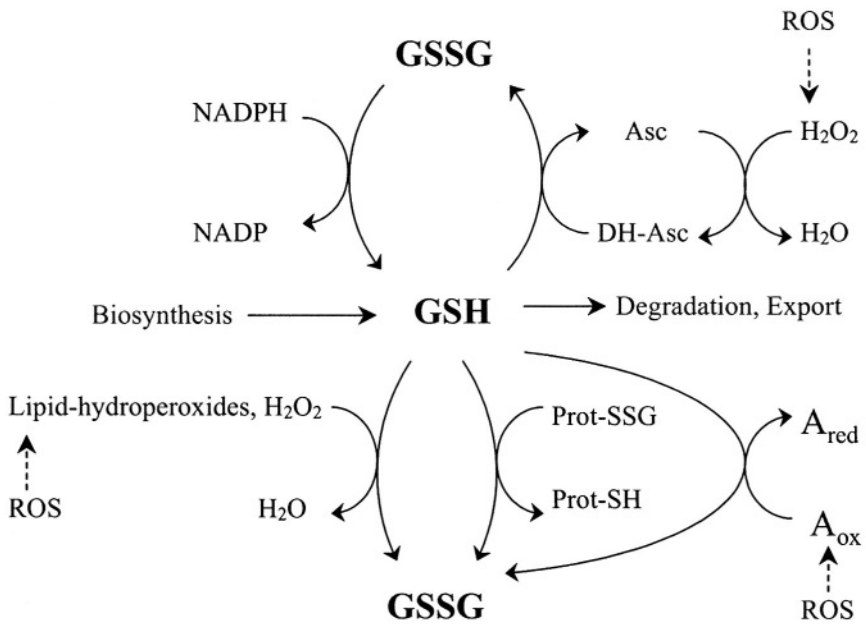

Figure 4. Comprehensive scheme of some possible roles of glutathione in protection against stress. A_{ox} = oxidized substrate, A_{red} = reduced substrate, Prot = protein, Asc = reduced ascorbate, DH-Asc = dehydroascorbate, ROS = reactive oxygen species, GSH = reduced glutathione, GSSG = oxidized glutathione.

(7) As a substrate of glutathione *S*-transferases (EC 2.5.1.18), glutathione is responsible for the removal of xenobiotics or other adverse organic compounds from the cytoplasm (see Rauser in this volume). Some GSTs are also

able to catalyse the reaction of GSH with products of lipid peroxidation chains and so function as repair enzymes in oxidative stress situations. If the peroxidation products are lipid hydroperoxides GSTs mimic a peroxidase activity.

In Figure 4, some of the complex roles of glutathione in antioxidative defence systems in plants are shown.

CHANGES IN THE GLUTATHIONE SYSTEM UPON STRESS IMPACTS

Numerous and partly contradicting reports exist on the responses of the glutathione system of plants to environmental stresses. A complete literature overview is not the intention of the present chapter. Some examples for the range of observations of the glutathione system related to environmental stress are given to illustrate the variability and the mechanisms of possible responses. For a more complete review see Alscher (1989), Polle and Renneberg (1994), Polle (1997), Noctor and Foyer (1998), Mullineaux and Creissen(1997).

The experiments conducted to understand the GSH system responses of environmentally stressed plants are based on three approaches: (1) Plants are transferred to a stress situation and the response is compared to non-stressed control, or plants under natural stress conditions are investigated in the field; (2) Variants, mutants, or cultivars with constitutive differences in their GSH metabolism are compared with respect to their stress tolerance; and (3) The most recent approach is the application of genetic engineering techniques (for more details see also Foyer and Noctor in this volume). Plants modified in specific aspects of their GSH metabolism are tested under stress situations.

Drought

Homoiohydric plants

Most higher plants are homoiohydric, which means they maintain a certain water status to avoid cell damage. These plants respond to water deficiency with stomatal closure to minimize water loss. If the restriction of the gas exchange is efficient in preventing water loss, a lack of CO_2 in the chloroplasts will occur. This leads to the situation of increased oxidative stress in illuminated chloroplast described in Figure 1 (Smirnoff 1993).

The drought effects on the glutathione system reported in the literature are dependent on the extent and intensity of the drought stress (via withholding water). Some studies found changes in the GSH system only under severe drought stress when relative water content was very low. At relative water contents below 40% an oxidation of the GSH pool as well as of the ascorbate pool was measurable in barley leaves (Smirnoff 1993). Although the plants recovered when re-watered immediately, the relative water content is near the survival limit for these species. This situation may reflect biochemical damages to the tissues rather than protective reactions. On the other hand, the initial responses to drought stress were investigated in a short-term study on *Pinus canariensis*, a drought resistant pine species, which is well able to prevent water loss by stomatal closure. The trees in this experiment did not exhibit visual damage or strong changes in needle water potentials. The first response to drought stress was a tight stomatal closure. Photo-protective changes in xanthophylls to dissipate excess light energy followed and, with a certain delay, the GSH/GSSG ratio dropped. Remarkably, these changes were accompanied by light dependent changes in total glutathione concentrations (Tausz et al. 2001). In contrast, drought stressed wheat cultivars showed an increased GSH/GSSG ratio together with a loss of total GSH. The more sensitive cultivar exhibited a corresponding increase in GR activity (Loggini et al. 1999). A loss of GSH and an increase in GR activity was also reported in osmotically-induced drought stress in rice hydrocultures (Boo and Jung 1999).

Desiccation tolerant plants

The situation is fundamentally different in desiccation tolerant plants. Only few higher plants, but many mosses, lichens, and algae are able to endure significant water loss by entering dormancy, a stage of very low metabolic activity. Upon wetting these poikilohydric plants, full life functions are quickly resumed. The poikilohydry is connected to the ability to maintain or quickly resume the active, reduced state of antioxidants during desiccation and resurrection. In *Tortula ruralis*, a poikilohydric moss, the glutathione pool oxidized and the activities of GR, GPX, and also GST increased if the moss was slowly dehydrated, but no changes were found upon rapid dehydration (Dhindsa 1987, 1991). Slowly dehydrated plants quickly reduced their glutathione pool upon wetting and the activities of the enzymes resumed control level. On the other hand, quickly dehydrated plants showed a transient increase in oxidized glutathione and the enzyme activities after wetting, and a subsequent return to control level within the next hours. A quick reduction within minutes of highly oxidized glutathione pools (70 to 90% of total) upon wetting were also found for some lichen species (Kranner and Grill 1997). The resurrection plant *Boea hygroscopica* (one of the

few higher plants that show poikilohydry) showed an increase in GSH contents upon dehydration, which was interpreted as a possible protection of proteins from oxidation (Navari-Izzo et al. 1997). In summary, an important trait connected with poikilohydry is the capacity to endure the high oxidation of the GSH pool and to quickly reduce it upon re-hydration.

Seeds

Many plants have poikilohydric resting stages, such as dormant seeds or spores. The dormancy of seeds and the loss of viability of recalcitrant (desiccation intolerant) seeds are accompanied by the action of ROS. Recalcitrant seeds seem to quickly lose their antioxidative protection (Hendry et al. 1992). As in poikilohydric plants, seed tissues have to reactivate their glutathione system when they resume physiological activity upon wetting. Observations on spores of *Neurospora crassa* and dormant seeds of *Triticum*, *Hordeum*, *Pisum*, and *Lycopersicum* all reported the same response (reviewed in Kranner and Grill 1997): The resting seeds contain highly oxidized glutathione pools, which were quickly reduced upon imbibition.

Light

Oxidative stress in the chloroplasts is generated by light energy, which drives the electron transport (Figure 1). It is not surprising that the light regime modulates the glutathione system in plant tissues. Sun-exposed spruce needles contained higher glutathione concentrations than shade needles of the same tree (Grill et al. 1987). Corresponding results were found among others in sun and shade leaves of the evergreen oak *Quercus ilex* (García-Plazaola et al. 1999a) and of five evergreen tree species of the Canarian laurel forest (Tausz, Rodríguez-Gonzalez, Wonisch, Jiménez, Morales, Grill, unpublished). Chloroplasts from pea leaves grown under high light conditions exhibited higher GR activities than those under low light (Gillham and Dodge 1987). However, GSH levels remained unchanged, a result also reported for sun and shade-grown beech leaves (Polle 1997). In etiolated wheat seedlings, a rapid increase of GSH within the first hours was observed upon illumination (Mattagajasingh and Kar 1989). In non-etiolated wheat seedlings a transfer from low to high light did not affect GSH concentrations, but GR activities increased transiently (Mishra et al. 1995). A corresponding result was previously reported by Foyer et al. (1989). In spruce needles, the glutathione content had a light dependent, diurnal course, with highest concentrations around midday (Schupp and Rennenberg 1988). This is consistent with the fact that glutathione synthesis itself is higher in illumi-

nated leaves (Noctor et al. 1997). The observed diurnal variations are at least partly due to a shift in the synthesis-export balance in the needles, since higher export rates were observed in the night (Schupp et al. 1992). Needles of *Pinus ponderosa* did not show changes in the GSH content when detached branches (with limited possibilities for glutathione export from the needles) were allowed to recover overnight from stressful conditions (Tausz et al. 1999). Also in whole plants, daily courses of the glutathione content in spruce needles were not found in all cases (Tegischer K., Tausz M., Wieser G., Grill D., unpublished). It seems possible that the potential of higher glutathione synthesis in the light is only activated under additional stress conditions. A recent study on spruce suggested that drought stressed trees exhibited light dependent changes in the GSH concentrations relative to controls (Tausz M., Monschein S., Wonisch A., Grill D., unpublished). An important regulating factor might be the supply of photorespiratory glycine, which may accumulate under stressful conditions in the light (Asada 1999). Glycine is a substrate for GSH synthesis and may be able to promote or limit the GSH formation (Noctor et al. 1999).

Since the reduction of GSSG by GR is dependent on NADPH, which is mainly provided by the photosynthetic electron transport, a light-dependence of the GSH/GSSG ratio might be expected. Some studies reported a more reduced glutathione pool in the light, but in other cases the GSH pool became more reduced during a dark period (Tausz et al. 1999). Because non-green tissues maintain their GSH redox pool in a reduced state, with the necessary reducing power probably supplied by the pentose phosphate cycle, a direct dependence of the GSH/GSSG redox ratio on the light regime is unlikely.

Low temperatures

Low temperature can induce different kinds of stress to plant tissues: (1) Photo-chilling is a situation where the Calvin cycle is slowed down or inhibited by low temperatures (depending on the plant species, often at 5 °C and below, higher for sensitive species such as maize), but absorbed light energy still drives the electron transport. This leads to the situation of an overproduction of ROS in the chloroplasts described in Figure 1. (2) Frost is detrimental to tissues mainly due the formation of ice crystals, which removes water from the liquid phase and causes damages to proteins and the ultrastructure of cells. The level of damage is dependent on the plant species, the developmental and phenological stage, the organ and tissues, and the degree of stress. ROS are also involved in freezing stress, participating in lipid per-

oxidation, protein destruction, and collapses of the antioxidant defence systems (Polle 1997).

Chilling has been shown to have a strong impact on the antioxidative systems, in particular in plants sensitive to chilling. Maize responds to chilling at temperatures as high as 11°C with an increased production of GSH and other antioxidants whereas the GSH/GSSG ratio dropped upon short-term chilling in young plants. Chilling-sensitive cultivars further increased their GSH concentrations upon long-term chilling and their GSH/GSSG ratios were lower than those of more tolerant lines (Hodges et al. 1996). Field experiments showed that the chilling tolerance of maize cultivars may be correlated to the capacity of increasing the GSH concentrations and GR activities under stress conditions (Leipner et al. 1999).

The cold tolerance of plant tissues is dependent on their physiological hardening stage. Under field conditions, a gradual adaptation to lower temperatures is accompanied by various physiological changes including increased antioxidant protection involving responses of the glutathione system which allow the plants to assume a new homoeostatic level (Polle 1997). Many studies reported increased GSH levels in winter, including spruce needles (Esterbauer and Grill 1978, Doulis et al. 1993), pine needles (Anderson et al. 1992), but also in winter wheat (Sagisaka et al. 1991) or in the living bark of apple trees (Kuroda and Sagisaka 1998). The signal for the increase in GSH concentrations seems to be the lower temperature and not the decrease of the light period length (Herbinger et al. 1999). Winter hardening is also marked by increased activities of GR in spruce needles (Esterbauer and Grill 1978, Doulis et al. 1993). Glutathione reductase activity in pea leaves was induced by cold treatment (Edwards et al. 1994). A direct causal connection between cold tolerance and GR activity was established by Foyer et al. (1995): in this study, transgenic poplar plants containing higher GR activity were significantly more cold tolerant than controls. In contrast, a direct causal connection of elevated GSH levels without increased GR activities to frost tolerance could not be established (Stuiver et al. 1995, Polle 1997). Contrasting results were found on the evergreen Mediterranean oak species *Quercus ilex*: in leaves of this species, glutathione concentrations did not undergo seasonal changes, but only GR activities increased in sun leaves during winter adaptation (García-Plazaola et al. 1999b).

High temperatures

Unlike low temperatures, high temperature effects on the glutathione system of plants have been studied less frequently. It has been shown that ROS

are involved in high temperature damage to plants, and excised maize roots strongly intensified GSH synthesis upon a heat shock of 40 °C (Nieto-Sotelo and Tuan-Hua 1986), but effects on the glutathione system of intact plants are largely unknown (Polle 1997).

UV radiation

Due to the shorter wavelength UV radiation transmits more energy per quantum than visible light and is potentially dangerous to organisms. The most important form of UV in the environment is UV-B (from 290 to 315 nm wavelength), because the UV-B doses are expected to increase due to the depletion of the protective stratospheric ozone layer. UV-A (315-400 nm) is only a minimal stress factor for plants (it is more important in the fuelling of atmospheric photo-smog reactions such as tropospheric ozone formation), whereas the highly destructive UV-C (220-290 nm) is quantitatively screened by the atmosphere. Plants respond with stress symptoms to elevated UV-B doses and ROS seem to be participate in these effects, too (Polle 1997). UV-B absorption may lead to the direct production of ROS at the cell surfaces (e. g. directly at the plasmalemma) and not only act by increasing the ROS production in chloroplasts like most other stress impacts.

Investigations of the GSH systems under UV stress are still rare. Cell cultures of *Rosa* responded to elevated UV-C (albeit ecologically irrelevant) with an oxidation of their GSH pool which resulted in enzyme inhibitions (reported in Polle 1997). In most experiments, the activities of glutathione-related enzymes (glutathione peroxidase and glutathione reductase) remained largely unaffected (Willekens et al. 1994, Rao et al. 1996), and also GSH concentrations did not increase in spruce upon exposure to near-ambient UV-B doses as a single stress factor (Polle 1997). More recently, results on maize suggested that UV-B does not change the pool size or redox state of GSH, but accelerates the turnover of this pool. In this species, increased GR activities were observed (Masi et al. 1995). Increased concentrations of cysteinyl-glycine, a low-molecular weight thiol, which is a possible degradation product of glutathione, pointed to an increased glutathione turnover (A. Masi, R. Ghisi, M. Feretti, unpublished results, Carletti 2000).

High elevations

High elevation imposes a combination of stress factors such as high irradiation (also UV-B irradiation) and lower temperatures, sometimes com-

bined with higher atmospheric ozone concentrations. The investigation of plants growing at high elevation sites provided information about the long-term adaptation to these stress conditions. Plants growing at high elevations often have higher constitutive glutathione concentrations, as found for spruce (Polle and Rennenberg 1992, Tausz et al. 1997), pine (Tausz et al. 1999), and some herbaceous alpine species (Wildi and Lütz 1996). Strong correlations of leaf and root concentrations of GSH with increasing altitude were also observed in *Plantago major* grown at elevations exceeding 3000 m (Ren et al. 1999). Such differences may be in part due to genetically fixed ecotypes. Spruce seedlings germinated from seeds collected at high elevations had higher foliar GSH contents than seedlings from seeds of lower elevation populations although they were grown together at a low elevated experimental site for several years (Guttenberger et al. 1992). On the other hand, environmental factors are also controlling the foliar GSH levels. A transfer of spruce seedlings from low to high elevations resulted in an increase of their needle GSH concentrations (Grill et al., 1988). However, contrasting results were also reported in the literature. At an elevation gradient from about 600 – 2000 m a.s.l. in the dry conditions on the southern slope of Tenerife Island, needles of field grown *Pinus canariensis* did not exhibit a clear elevation dependence of their glutathione concentrations (Tausz et al. 1998). When different clones of spruce trees were planted along a gradient from 700-1750 m in the Alps, changes in the constitutive glutathione concentrations of the needles were not found. Only trees of one clone which were found particularly sensitive to ozone-induced oxidative stress lost GR activity under high elevation conditions (Polle et al. 1999).

Salinity

Salinity is a common stress plants experience and may act in multiple ways: (1) By ability to inhibit enzymes of the Calvin cycle, salinity induces oxidative stress in plant cells according to the scheme in Figure 1.; and (2) Due to changes in the osmotic balance, high salinity can cause water loss in cells and induce drought stress in the plant tissues.

Responses of the glutathione systems to salt exposure have been reported: For example, in *Arabidopsis thaliana*, GSH and also cysteine levels increase markedly upon exposure to salt stress (Barroso et al. 1999), indicating a strongly induced GSH synthesis. In rice, salinity caused an induction of GR (Kaminaka et al. 1998). In the latter case, this induction was apparently connected to the hormonal stress response of plants, namely to the synthesis of abscisic acid, which is also related to drought and chilling stresses. The GPX

response demonstrated in *Citrus* is also induced by salt stress, possibly directly as a result of the stress-induced formation of phospholipid-hydroperoxides (the best substrate for this enzyme) which occurred only in less tolerant cells (Avsiankretchmer et al. 1999).

A short summary of responses of the glutathione system to natural stress

In general, many studies revealed increases in glutathione reductase activities and/or the total glutathione concentrations upon the impact of stresses. Such a response is coincident with a re-enforcement of the antioxidative defence systems and generally interpreted correspondingly. With more severe stress, a degradation of total GSH concentration was reported which suggested a weakening of the defence system as a symptom of initial cell disruption. However, the responses of the GSH/GSSG redox state are more divergent. Oxidation of the GSH pool was regarded as a symptom of initial collapse of the antioxidant system as may be true in severe cases, but this redox ratio may also play an important role in detecting the stress level and signalling further responses (May et al. 1998). Small and transient changes in the GSH/GSSG ratio preceding other responses may be possibly be interpreted in this way. Hence, the regulation aspects of the glutathione metabolism and the antioxidant system as a whole are also of great importance.

REGULATORY ASPECTS OF THE GLUTATHIONE SYSTEM UNDER STRESS IMPACT

With respect to the regulative function of glutathione in plant cells under stress, two main aspects may be addressed: (1) The regulation of the glutathione system itself under stress conditions; and (2) the regulatory potential of the GSH system toward other components of the cellular defence.

A change in the GSH/GSSG redox ratio can be observed in response to many stress factors. This ratio is potentially able to influence *in vivo* enzyme activities by protein-SH/GSH interactions (Kunert and Foyer 1993). Some experiments illustrate different mechanisms of the regulatory role of the GSH redox system in stress responses. Enzymes of the lignin-synthesis pathway, which were involved in plant defence reactions, were shown to increase from both the mRNA and the enzyme activity level (Wingate et al. 1988). In an experiment where GSH was fed to detached pine shoots, higher

GSH/GSSG ratios depressed the concentration of CuZn-SOD mRNAs in the cytosol. That means that the expression of this antioxidative enzyme is derepressed under oxidizing conditions. However, the SOD activity itself and the isozyme composition of SOD remained unchanged. A lower GSH/GSSG ratio increased the activity of glutathione reductase, but not the mRNA concentrations attributed to this enzyme. Glutathione seems to have a redox control over the expression of the investigated SOD, and to regulate GR on the translation or the protein level (Wingsle and Karpinski 1996). It is possible that only some isoforms of GR are directly controlled by GSSG itself (Mullineaux and Creissen 1997). Due to these contrasting results, the role of GSH in the regulation of cellular defence systems is still unclear (Polle 1997), although the idea of a transient change in the GSH/GSSG ratio as a common start signal for many cellular defence actions is intriguing (see also Foyer and Noctor in this volume).

The control of glutathione pool and the glutathione redox state itself seems to be even more complicated (Figure 5). Glutathione synthesis is apparently under redox control, possibly by GSH/GSSG ratio itself. In *Arabidopsis*, the translation of protein for γ-GC synthetase, the rate limiting enzyme for GSH synthesis, is repressed by high GSH/GSSG ratios. (Xiang and Betrand 2000). Furthermore, sulphur assimilation into cysteine as a requirement for GSH synthesis can also be oxidatively induced. For example, the expression rates and the activities of the key enzymes APS-reductase and ATP-sulphurylase (but not cysteine-synthetase) increased upon a decreased GSH/GSSG-ratio in maize roots. This is even more remarkable because most enzymes of assimilation pathways in the chloroplasts are induced under the reducing conditions of a highly active light-driven electron transport (Flückinger et al. 2000). ATP-sulphurylase activity increased in canola roots upon mild oxidative stress, which also caused a drop in the GSH/GSSG ratios (Lappartient and Tourraine 1997). However, some of these enzymes were also controlled by sulphur starvation, by stress-related plant hormones such as jasmonic acid, and by heavy metals which also require increased GSH for their detoxification (see Rauser in this volume). In some cases the oxidative regulation differs from other regulation impacts with respect to co-regulated partner enzymes.

OUTLOOK

Although significant progress has been made in understanding glutathione metabolism and regulation chiefly via the introduction of molecular techniques and genetically transformed plants, the results obtained with respect to overall stress responses are still extremely variable. Plants with

higher constituent GSH levels or GR activities may or may be not more resistant (Strohm et al. 1995, Aono et al. 1991) or even more susceptible to different oxidative stresses (Creissen et al. 1999) when the whole plant system is observed. It must be taken into account that glutathione is only one, albeit important, constituent of the defence and regulating system in plants.

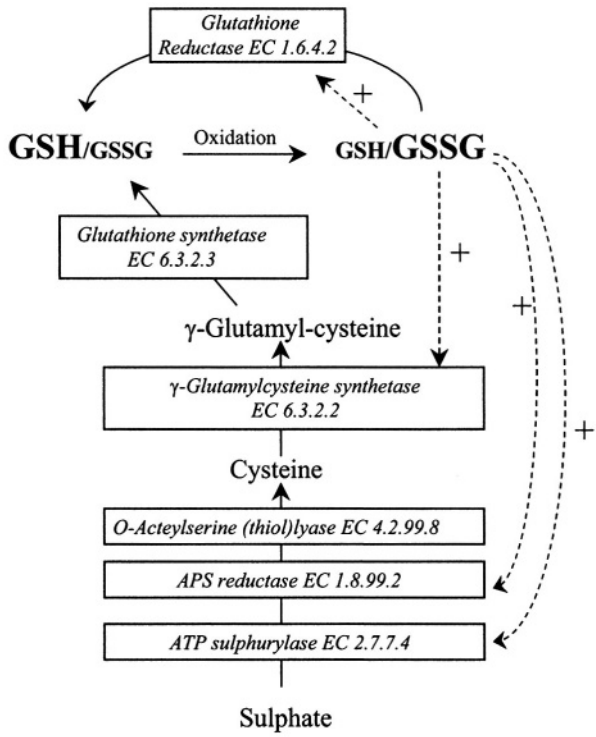

Figure 5. Possible redox regulation mechanisms of the glutathione system. The dotted arrows indicate oxidative up-regulation. Note that not in all cases it the direct regulation of enzyme activity by a high GSSG/GSH-ratio is established. A simultaneous effect of oxidizing conditions on both enzyme activities and glutathione redox system is also possible. The sulphur assimilation pathway is not drawn in all details!

More crucial than the concentration, redox state, or enzyme activity of specific parts of this system may be the concerted action of all parts. In some cases, under particular conditions, and in certain species, the controlled response to an environmental stress may require direct responses of the glutathione systems, whereas in other cases or in other species it may not. The

strategies applied to mitigate, avoid or counteract stress effects may be quite different even within the same species, which makes the overall interpretation of data difficult. To enlighten these differences, not only studies in more detail on the molecular level are required, but also approaches that investigate whole plant systems under different environmental conditions, different stress intensities, and include the temporal scale of the response. Multivariate evaluations of multiple constituents of the antioxidant system better represent the concerted action of the whole system (Tausz et al. 1998). Furthermore, the differential response between organs, tissues, cells, and subcellular compartments toward stress is currently largely unknown, although the major importance of trans-organ, inter-cellular and intra-cellular signalling is beyond doubt. Only recently, microscopic techniques involving fluorescence microscopy and image analysis (Müller et al. 1999, image on the front cover) or laser-scanning microscopy (Sanchez-Fernandez et al. 1997) allowed the observation of subcellular GSH pools.

The integration of different investigation methods on different organization levels from the gene expression to the whole plant regulation promises further insight in the complex roles of glutathione in plant stress responses.

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Chapter 6

THE ROLE OF GLUTATHIONE IN PLANT REACTION AND ADAPTATION TO EXCESS METALS

Wilfried E. Rauser

INTRODUCTION

Plants growing in soils and aquatic environments are exposed to essential micronutrients such as Cu and Zn and perhaps nonessential elements including Cd, Hg and Pb. These metals exist at low to high concentrations depending on variations in natural habitats and manmade disturbances. Plant growth and survival in a fluctuating nutrient environment are enhanced when mechanisms operate to maintain internal concentrations of essential metals between deficient and toxic limits and keep nonessential metals below their toxicity thresholds. Since 1984 several groups of small cysteine-rich peptides have been discovered that provide sulphydryl or thiol groups for sequestering certain metals. Copper, Cd, Hg, Pb and Zn are bound particularly because they share an affinity for thiol groups (Nieboer and Richardson 1980). The hypothesis is that cysteine-rich peptides participate in cellular homeostasis and detoxification. For plants and certain fungi glutathione is central to the formation of cysteine-rich peptides.

Present knowledge in this field rests extensively on plant reactions to Cd largely because of the concern for Cd entry into the food chain of animals and man (Wagner 1993). The developing field of phytoremediation applied to metal polluted sites (Salt et al. 1998) has some foundations in the specific metabolism of glutathione considered in this chapter. The situation for plants is considered most extensively with some reference to certain yeasts that have provided critical and relevant information. The treatment of the subject reflects the author's limitations and views of the area. More extensive reviews and overviews on metal-binding molecules in plants are available (Rauser 1990, Steffens 1990, Robinson et al. 1993, Prasad 1995, Rauser 1995, Zenk 1996).

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MONOTHIOL AND METAL-INDUCED POLYTHIOL PEPTIDES

Glutathione and related monothiol peptides

Three monothiol peptides are well known in plants. Glutathione (γGluCysGly) is the tripeptide central to diverse plant functions as described in this volume. In certain members of the Fabaceae homoglutathione (γGluCysβAla) is the homologue that partly or completely replaces glutathione. The biosynthesis of glutathione and homoglutathione depends on the third monothiol γ-glutamylcysteine (γGluCys) (Macnicol 1987). Glutathione, aside from homoglutathione in certain species, is often the most abundant monothiol peptide in roots and shoots of normal young plants. The contents of γGluCys are generally much lower and can be exceeded by cysteine (Meuwly and Rauser 1992, Rüegsegger and Brunold 1992).

A fourth monothiol, and homologue of glutathione, is γ-glutamylcysteinylserine (γGluCysSer). This tripeptide occurs in some members of the family Poaceae (=Gramineae) (Klapheck et al. 1992). It was more abundant in leaves than in roots and in most cases was less abundant than glutathione. In no species did γGluCysSer replace glutathione. Out of the 32 genera analyzed *Eragrostis*, *Saccharum*, *Sorghum* and *Zea* contained no γGluCysSer. Species from other families require analysis. Biosynthesis of γGluCysSer is unclear. Inhibition of the enzyme γ-glutamylcysteine synthetase by buthionine sulphoximine caused nearly equal reductions of γGluCysSer and glutathione contents, as if biosynthesis of the seryl tripeptide was connected to γGluCys as for glutathione and homoglutathione (Klapheck et al. 1992). However, these workers could not exclude the possibility that carboxy-terminal glycine might be hydroxymethylated in a reaction similar to the synthesis of serine during photorespiration.

The fifth monothiol, and homologue of glutathione, is γ-glutamylcysteinylglutamic acid (γGluCysGlu). This tripeptide was found in maize seedlings after exposure to Cd (Meuwly et al. 1993), its presence in other plants has not been assessed. It appeared in roots after two hours of Cd exposure, in shoots after two days. The contents of γGluCysGlu were consistently less than those of glutathione. Little is known about the origin of γGluCysGlu. Its restriction to maize tissues exposed to Cd and after polythiol peptides have formed (see below) suggested a catabolic source. Cleavage of intramolecular γGlu linkages by a γ-glutamyl transpeptidase would be required (S. Klapheck, personal communication). The influence of

buthionine sulphoximine on γGluCysGlu contents was not evaluated principally because the tripeptide was absent in control maize tissues and it appeared after glutathione contents began to fall with Cd exposure.

A sixth monothiol, γ-glutamylcysteinylglutamine (γGluCysGln), was tentatively assigned through spiking the supernatant fraction of Cd-exposed hairy roots of horseradish with chemically synthesized γGluCysGln (Kubota et al. 2000). Complete identification of the monothiol was not made because of its low abundance in the tissue. This monothiol was anticipated after the structure of Cd-induced polythiols with carboxy-terminal Gln were characterized by mass spectrometry (see below).

The six monothiol peptides mentioned so far are listed in Table 1. They have two common features: the γ-carboxyamide linkage from Glu to Cys, and the provision of thiol by Cys. Due to the affinity of the metal ions Cu, Cd, Hg, Pb and Zn for thiols, these six monothiols may function as initial metal-sequestering molecules. The cytosol of roots is the first cellular compartment reached by metals in most environments. The cytosol is one location of the synthetases for γ-glutamylcysteine and glutathione (Rüegsegger and Brunold 1993) and so provide monothiols for local function.

Table 1. The various mono- and polythiol peptides found in plants exposed to excess cadmium. The thiols are listed in the order that they were found as Cd-induced peptides. Trivial names are attributed to the originators through the citations in the footnote.

Amino acid sequence in the monothiols	polythiols	trivial name	A proposed Nomenclature [1]
1. γGluCysGly		glutathione	
	$(\gamma GluCys)_nGly$	cadystin [2], phytochelatin [3]	Phytochelatin (PC)
2. γGluCysβAla		homoglutathione [4]	
	$(\gamma GluCys)_n\beta Ala$	homophytochelatin [5]	*iso*-PC(βAla)
3. γGluCys		desGly peptide [6]	
	$(\gamma GluCys)_n$	desglycine phytochelatin [1]	desGly(PC)
4. γGluCysSer		hydroxymethyl-glutathione [7]	
	$(\gamma GluCys)_nSer$	hydroxymethyl-phytochelatin [7]	*iso*-PC(Ser)
5. γGluCysGlu		none	
	$(\gamma GluCys)_nGlu$	none	*iso*-PC(Glu)
6. γGluCysGln		none	
	$(\gamma GluCys)_nGln$	none	*iso*-PC(Gln)

[1] Zenk 1996; [2] Kondo et al. 1984; [3] Grill et al. 1985; [4] Carnegie 1963; [5] Grill et al. 1986a; [6] Mehra and Winge 1988; [7] Klapheck et al. 1994.

Polythiol peptides

The polythiol peptides in Table 1 belong to a group of molecules designated metallothioneins. These are polypeptides sharing a low molecular mass, high cysteine content with absence of aromatic amino acids and histidine, high metal content, an abundance of CysXaaCys sequences where Xaa is an amino acid other than cysteine, spectroscopic features characteristic of metal thiolates, and metal thiolate clusters. Metallothioneins are now subdivided into three classes based on their structure (Fowler et al. 1987).

Class I: polypeptides with locations of cysteine closely related to those in equine renal metallothioneins;

Class II: polypeptides with locations of cysteine only distantly related to those in equine renal metallothioneins;

Class III: atypical, nontranslationally synthesized metal thiolate polypeptides.

The polythiol peptides in Table 1 are class III metallothioneins because ribosomal synthesis produces only α-carboxyamide bonds and not the γ-carboxyamide linkage. It follows that no genes directly define the primary structure of the polythiol peptides, rather gene-controlled non-ribosomal enzyme(s) are involved. The polythiols are structurally related to the respective monothiols, whether a biosynthetic relationship exists remains to be defined for most polythiols. No concensus is available concerning trivial names for the polythiols. The family with a carboxy-terminal Gly was first characterized for the fission yeast *Schizosaccharomyces pombe* exposed to Cd, the two peptides produced were called cadystins A and B (Kondo et al. 1984). Shortly thereafter a larger series within the same peptide family was found in several plants exposed to various metals (Grill et al. 1985). The name phytochelatin was proposed because this series of polythiols was not restricted to Cd nor to fungi. Since phytochelatins had a structural relationship to glutathione, the homologues related to homoglutathione were called homophytochelatins (Grill et al. 1986a) and those related to hydroxymethylglutathione were designated hydroxymethyl-phytochelatins (Klapheck et al. 1994) (Table 1). In a recent review, Zenk (1996) proposed a unified nomenclature based on phytochelatin (PC). Those peptides having a carboxy-terminal amino acid other than Gly are named *iso*-phytochelatin (*iso*-PC) with the parenthetic addition of the carboxy-terminal amino acid abbreviation [e.g. *iso*-PC(βAla)]. The listing for the six families of polythiol peptides is given in Table 1. This new terminology leaves room for new *iso*-PCs should they be discovered (Zenk 1996). The prefix *iso* was chosen to signify the equal function of the peptides. In this chapter the polythiol peptides are collectively called γGluCys peptides based on the common structural ele-

ment. Specific thiols are named according to the sequence of amino acids (Table 1). Since most specific biochemical data are confined to the $(\gamma GluCy)_nGly$ family the original name phytochelatin is used to ease consultation with original articles.

Peptide analysis

To determine the metabolic relationships between glutathione and the various γGluCys peptides and to elucidate peptide functions in cells it is necessary to quantify individual polythiols. Source materials vary from yeasts to algae, cultured plant cells, and roots and shoots of metal-exposed plants. General protocols for isolating metal-binding complexes and quantitating γGluCys peptides have been described (Grill et al. 1991, Hayashi et al. 199la, Mutoh and Hayashi 1991, Rauser 1991) and current papers may have specific adaptations.

Acid extraction of the source material with 100 mM HCl or 5 % (w/v) 5-sulfosalicylic acid solubilizes the peptides and precipitates some proteins. At the same time, however, metal-thiol coordination is destroyed and the signature function of metal-binding is lost. The acid extract is analyzed by high performance liquid chromatography (HPLC) using a C_{18} reverse phase column. The mobile phases are acidic, either 0.05 % or 0.1 % (v/v) trifluoroacetic acid, and a linear gradient from 0 to 20 % (v/v) acetonitrile over 40 minutes is used to elute the specific peptides. Components in the column effluent may be monitored for the peptide bond at 220 nm (Figure 1A). This may be suitable for extracts from pre-purified metal-binding complexes or enzyme assays. It is not suitable for crude extracts because some peaks are difficult to discern and UV-absorbing materials other than thiols confound peak areas. Specific monitoring of thiol is superior (Figure 1B). Continuous addition of Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid); DTNB) to the column effluent allows the DTNB to react with any sulphydryl groups and release the thionitrobenzoate anion that absorbs broadly at 412 nm. Using a more concentrated Ellman's reagent delivered at a low flow rate is recommended to minimize changes in resolution of closely eluting γGluCys peptides. Under such conditions effective micro mixing of reagent and column effluent is essential. A residence time of at least 1.2 minutes in a narrow, long post column reaction coil ensures complete reaction of all sulphydryls in the longer oligomers of γGluCys peptides. In these circumstances the colour generated by a standard quantity of glutathione provides a suitable calibration for thiol irrespective of their number in a peptide. The shorter post column reaction time of 6 seconds used by Gupta and Goldsbrough (1991)

necessitated unique calibrations for the individual phytochelatins with n=3 through 6. In our laboratory fairly large quantities of thiols are applied to the HPLC column with the knowledge that the response for any individual thiol is linear up to 50 nmol thiol/peak (Rauser 1991). In this way major components can be quantified and minor, yet unidentified, thiol constituents are evident (Figure 1B). The peak detection threshold with DTNB is about 0.2 nmol thiol.

In the case of microalgae and foliage from natural environments more sensitive methods are required. The thiols in acid extracts are derivatized with monobromobimane and the biname-labelled thiol peptides are separated by reverse phase HPLC. Measuring the fluorescence of the bimane adduct allowed detection of low picomol quantities of thiol (Ahner et al. 1995, Gawel et al. 1996, Rijsteribil and Wijnholds 1996). Synthetic standards of individual phytochelatins were used to calibrate fluorescence signals. In the author's experience the fluorescence yield per mol thiol for a variety of monothiols was the same, however, the yield declined progressively for phytochelatins n=2 through 4. Either the kinetics of thiol derivatization with monobromobimane decreased progressively with the polythiols or there was greater self-quenching of fluorescence as the number of fully derivatized Cys increased in the molecules. This highly sensitive technique may serve as a qualitative measure, for quantitative analyses separate calibration curves are necessary for each polythiol.

Determining structures of γGluCys peptides

Individual thiols purified by HPLC can be used to determine the amino acids present according to standard procedures including performic acid oxidation of Cys. In this way a variety of polythiols have been shown to contain nearly equimolar amounts of Glx and Cys and lesser quantities of Gly, Ser or βAla. For two thiols from maize Glx substantially exceeded Cys (Meuwly et al. 1995). Acid hydrolysis does not allow differentiation between Glu and Gln in the original peptides. Although this procedure does not provide the sequence of amino acids, in new investigations it can be corrobatory evidence for identifying putative peaks as specific γGluCys peptides.

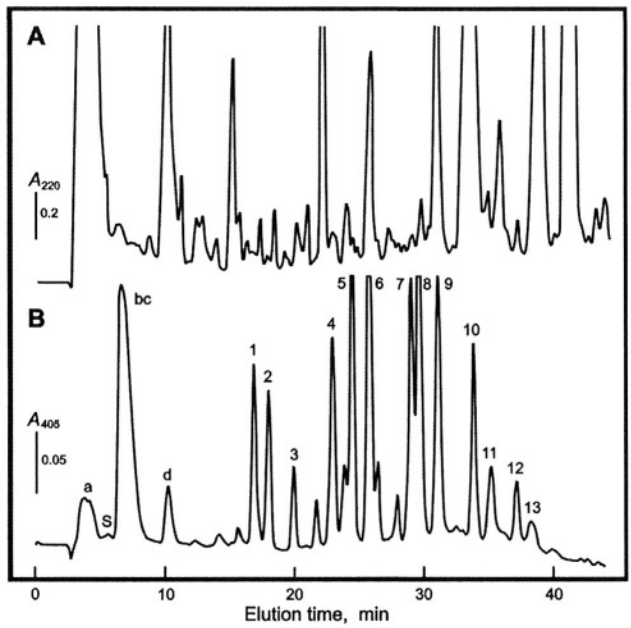

Figure 1. HPLC chromatograms of crude acid soluble material from roots of maize seedlings exposed to 3 μM Cd for 6 days. One mL of acid extract equivalent to 0.513 g fresh mass of maize (Cargill hybrid 37701) was applied. The column was developed with a linear gradient from 0 to 20 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid over 40 minutes (Rauser 1991). The column effluent was first monitored for A_{220} in panel **A** followed by continuous addition of Ellman's reagent to give the thiol profile in panel **B**. The early components in panel **B** are: a, cysteine unresolved from breakthrough material; S, the acid labile sulphide not volatilize; bc, glutathione and γGluCys, respectively, unresolved; d, γGluCysGlu. The numbered peaks are the polythiols in three families: (a) phytochelatins: peak $1 = n_2$, $5 = n_3$, $8 = n_4$, $11 = n_5$; (b) $(\gamma GluCys)_n$: $2 = n_2$, $6 = n_3$, $9 = n_4$, $12 = n_5$, 4 = a variant of n_3; (c) $(\gamma GluCys)_nGlu$: $3 = n_2$, $7 = n_3$, $10 = n_4$, $13 = n_5$.

A variety of methods have been used to sequence the amino acids. The peptides are not sensitive to Edman degradation because γ-carboxyamide bonds are not cleaved. The earliest method relied on chemical modifications and digestion with carboxypeptidase P (Kondo et al. 1984). The structures of $(\gamma GluCys)_2Gly$ (cadystin A) and $(\gamma GluCys)_3Gly$ (cadystin B) were confirmed by chemical synthesis. Grill and co-workers (Grill et al. 1985, Gekeler et al. 1988) treated S-benzylated peptides with γ-glutamyltranspeptidase, determined the amino acid liberated, then applied a modified Edman degradation to the residue and determined the amino acid liberated. Cyclical repetition of the enzymatic and chemical steps gave the complete sequence of phytoche-

latins $(\gamma GluCys)_nGly$ where n=2 to 5 depending on the peptide analyzed. Jackson et al. 1987 used enzymatic digestion and NMR spectroscopy to determine the primary structure of phytochelatins n=2 and 3 from cultured cells of *Datura innoxia.* Mass spectrometry was first applied to phytochelatins from cultured cells of tomato by Steffens et al. (1986). The (M+H) ion of a peptide was selected and bombarded with argon atoms to cause fragmentation largely at the amide linkages. Two peptides had the sequence $(\gamma GluCys)_3Gly$ and $(\gamma GluCys)_4Gly$, deuterium exchange experiments showed the γ-carboxyamide bond between Glu and Cys. Various techniques in mass spectrometry have been used to sequence phytochelatins from the fission yeast *S. pombe* (Kon-Ya et al. 1990) and brewer's yeast *Saccharomyces cerevisiae* (Kneer et al. 1992), $(\gamma GluCys)_3Ser$ from rice (Klapheck et al. 1994), and $(\gamma GluCys)_{2\text{-}4}Gly$, $(\gamma GluCys)_{2\text{-}4}$ and $(\gamma GluCys)_{2,3}Glu$ from maize (Meuwly et al. 1995). Amino terminal γGlu could be proven through the properties of three low mass ions, internal Glu moieties were assumed to be bonded via the γ-carboxyl group because no αGlu was detected and the peptides were not cleaved by endoprotease αGlu-C from *Stephalococcus aureus* V8. In the work with maize the DTNB post column reaction products were collected and purified for mass spectrometry. The assumption that the thionitrobenzoate adduct would protect the sulphur atom of Cys did not hold when two contiguous Cys were present in the molecule, rather a disulphide bond formed uncontrollably (Meuwly et al. 1995). This complexity was not encountered when non-derivatized peptides were analyzed (e.g. Klapheck et al. 1994). Isolation of two thiol peaks from hairy roots of horseradish and their characterization through electrospray ionization-mass spectrometry established $(\gamma GluCys)_3Gln$ and $(\gamma GluCys)_4Gln$ (Kubota et al. 2000).

The variety of γGluCys peptides known are currently not available from vendors of peptides. This is a difficulty for new investigators in calibrating their HPLC profiles. An enzymatic method using crude homogenate from *Silene vulgaris* suspension cells yields gram quantities of phytochelatins with n=2 through 5 (Friederich et al. 1998). If new HPLC profiles match published data then amino acid analysis of putative γGluCys peptides and exact mass determinations (e.g. Kubota et al. 1995, 2000) provide the necessary corroboratory identification. Co-chromatography of chemically synthesized peptides is further evidence. If HPLC profiles do not match, unassignable peptides require purification and sequencing perhaps by mass spectrometry. For the primary sequences given above some higher order oligomers present in low amounts in acid extracts have been identified in HPLC profiles without chemical analysis. This was done by applying the rule that retention times of peptides during linear HPLC gradients increase

logarithmically (Sasagawa et al. 1982). Gekeler et al. (1989) used the relationship:

$$\text{Retention time phytochelatin}_n - \text{Retention time glutathione} = a \cdot \log n$$

for phytochelatins n=2 through 6. With this relationship they identified phytochelatins with n=7 through 11 in *Rauvolfia serpentina.* Unique relationships were also found for each of the three families of γGluCys peptides present in maize (Meuwly et al. 1995).

Induction of γGluCys peptides by metals

Various metals cause the appearance of γGluCys peptides in plants (Table 2). For four studies single concentrations of metals ranging from 10 to 100 μM were used, in others comparisons between metals were based on a series of concentrations (e.g. tomato cells, *Rubia tinctorum* roots, a marine diatom). Environmentally realistic metal concentrations in the realm,of 1 pM to 100 nM were used by Ahner and Morel (1995) in their study with the marine diatom *Thalassiosira weissflogii.* The metals in Table 2 are grouped according to the classification of Nieboer and Richardson (1980). Silver through Cu(I) are class B metal ions that seek out nitrogen and sulphur centres in biological systems. Copper(II) through Zn are borderline metals that can form stable complexes with ligands offering oxygen, nitrogen or sulphur atoms. Selenate, Te and W are outside the groupings suggested by Nieboer and Richardson. All metals induced phytochelatins except in those members of the Fabales without glutathione where homophytochelatins appeared (Table 2). In cultured roots of *R. tinctorum* phytochelatins were accompanied by the $(\gamma GluCys)_n$ family except with Ni and Se. Initial reports of inductions by Ni, Se, Te and W could not be repeated perhaps reflecting analytical problems at low phytochelatin levels (Zenk 1996). The abundance and length of the induced γGluCys peptides varied with the metal used. In some cases a somewhat effective metal at one concentration could not be tested at higher concentrations because its toxicity killed the cells. Cadmium is generally considered the most effective inducer of phytochelatins but was surpassed by Ag in *R. tinctorum* roots. No phytochelatin induction was found for Al, Ca, Cr, Cs, K, Mg, Mn, molybdate, Na or V (Grill et al. 1987). Aside from Cr and Mn that are borderline metals, the foregoing list of metals are all class A metals that share a strong preference for ligands with oxygen as the donor atom.

Table 2. Induction of γGluCys peptides by metals and anions in different organisms. Empty entries signify that the element or anion was not tested. Phytochelatins were found in all cases except those of the Fabales where homophytochelatins were induced. The class B metal ions start with Ag and end at the first dashed lines with Cu(I), the borderline metal ions start with Cu(II) and end with Zn, the last three elements are outside the groupings considered by Nieboer and Richardson (1980).

	Rauvolfia serpentina [1]	tomato [2]	*Rubia tinctorum* [3]	fission yeast [4]	*Scenedesmus Chlorella* [5]	a marine diatom [6]	plants of the Fabales [7]
Ag	+	+	+	+	+	+	+
Au	+	+					
Bi	+			+			
Hg	+	+	+		+	+	+
Pb	+	+	+	+	+	+	+
Cu	+	+	+	+	+	+	
As	+		+	+			+
Cd	+	+	+	+	+	+	+
Co						+	
Fe		+					
Ga			+				+
In			+				
Ni	+	+	+			+	
Pd			+				
Sb	+						+
Sn	+						
Zn	+	+	+	+	+	+	+
Se	+		+				
Te	+						
W	+						

[1] Grill et al. 1987; [2] Chen et al. 1997; [3] Maitani et al. 1996; [4] Grill et al. 1986b; [5] Gekeler et al. 1988; [6] Ahner and Morel 1995; [7] Grill et al. 1986a.

Distribution of γGluCys peptides amongst plants and fungi

Phytochelatins occur in all the organisms tested that produce glutathione. These range from the smallest algae (Ahner and Morel 1995, Gekeler et al. 1988) through the non-vascular plants to mono- and dicotyledonous plants (Gekeler et al. 1989). Amongst 43 species in the Family Fabaceae (=Leguminosae) 7 produced only phytochelatins, 13 only homophytochelatins and 23 made both types of γGluCys peptides (Grill et al. 1986a). The type of peptide found depended on the monothiol produced by individual species; whether only glutathione, only homoglutathione or both. The fission yeast *Schizosaccharomyces pombe* produces $(\gamma GluCys)_nGly$ and $(\gamma GluCys)_n$ in response to Cd and Cu (Mehra and Winge 1988) yet *Candida glabrata*

produces the γGluCys peptides with Cd and class II metallothionein with Cu (Mehra et al. 1988). Other fungi known to contain class II metallothionein, *Saccharomyces cerevisiae* and *Neurospora crassa,* are able to produce phytochelatins when challenged with Cd (Kneer et al. 1992). These authors list other yeasts and Basidiomycetes containing phytochelatins. The $(\gamma GluCys)_nSer$ peptides have been found in six members of the Poaceae along with phytochelatins and $(\gamma GluCys)_n$ (Klapheck et al. 1994). Maize is the only plant so far that has $(\gamma GluCys)_nGlu$ peptides accompany phytochelatins and $(\gamma GluCys)_n$ (Meuwly et al. 1995), horseradish is the only plant so far that has $(\gamma GluCys)_nGln$ peptides accompanying phytochelatins (Kubota et al. 2000).

GLUTATHIONE AND BIOSYNTHESIS OF POLYTHIOL PEPTIDES

Discovering the primary structure of phytochelatins as compounds with repeating γ-carboxyamide linkages led to a paradigm shift away from gene control of peptide synthesis on ribosomes to enzymatic biosynthesis. The constancy of γGluCysGly in the carboxy terminus of each member of the phytochelatin family suggested a biosynthesis dependent on glutathione. This hypothesis is now supported in various ways. The situation for the other families of γGluCys peptides remains unclear. Whether specific biosyntheses are simply a dependence on the availability of monothiols (e.g. glutathione versus homoglutathione in certain Fabales) is unclear. In other plants the origins of the pertinent monothiols γGluCysSer, γGluCysGlu and γGluCysGln are unclear. Does their biosynthesis parallel that of glutathione or are they degradation products of polythiols originally derived from glutathione?

Involvement of glutathione in phytochelatin biosynthesis

The kinetics of phytochelatin appearance are particularly illuminating (Figure 2A). Cells of *R. serpentina* were grown in Zn- and Cu-free medium for three days prior to addition of 200 μM Cd (Grill et al. 1987). Rapid formation of $(\gamma GluCys)_2Gly$ occurred, after increasing lag times the n=3, 4 and 5 oligomers appeared. The cellular pools of glutathione declined during the first three hours of phytochelatin synthesis nearly in proportion to the amount of γGluCys incorporated. These data and a similar result for fission

yeast (Grill et al. 1986b) suggested that the phytochelatins were synthesized by sequential addition of γGluCys residues first to glutathione and then to the growing phytochelatin chain. Appearance of phytochelatin within 30 minutes suggested that the enzyme(s) involved in biosynthesis occurred constitutively. The apical 10 cm region of roots of maize seedlings exposed to 3 μM Cd showed kinetics similar to those in Figure 2A (Tukendorf and Rauser 1990). Phytochelatin n=2 occurred initially at a low background level because the plants were grown in nutrient solution containing Cu and Zn. Within 15 minutes of Cd addition the n=2 peptide increased and the n=3 and 4 oligomers appeared later. Use of buthionine sulphoximine to inhibit γ-glutamylcysteine synthetase effectively suppressed phytochelatin synthesis *in vivo* to show that γGluCys, perhaps through glutathione, was essential for phytochelatin production (Grill et al. 1987, Scheller et al. 1987). Four mutants of *S. pombe* that only synthesized low levels of glutathione were Cd-hypersensitive because they could not form sufficient phytochelatins to sequester the metal (Mutoh and Hayashi 1988). The *cad-2* mutant of *Arabidopsis thaliana,* deficient in glutathione for lack of glutathione synthetase, produced few phytochelatins and was Cd-hypersensitive (Howden et al. 1995b).

Addition of exogenous glutathione to tomato cells during exposure to Cd enhanced their ability to produce phytochelatins n=2, 3 and 4 (Mendum et al. 1990). Elevation of endogenous glutathione in maize roots with dichlormid (N,N-diallyl-2,2-dichloroacetamide), a herbicide safener, also enhanced phytochelatin production on Cd exposure (Tukendorf and Rauser 1990). Early loss of glutathione upon addition of Cd (Figure 2A) is documented for cells and tissues of various species. Full recovery of glutathione pools in roots of maize and rice to levels in controls was not evident even after seven days of exposure (Meuwly and Rauser 1992, Rüegsegger and Brunold 1992, Klapheck et al. 1994). Perhaps the lower glutathione level is a biological strain consequent to the stress of the altered metabolism caused by excess Cd. Overexpression of bacterial glutathione synthetase in the cytosol of Indian mustard caused a 5-fold increase in glutathione and a 1.7-fold increase in phytochelatin n=2 in roots exposed to 100 μM Cd over that in wild-type plants (Zhu et al. 1999). The growth inhibitory effects of 50 and 100 μM Cd on wild-type plants were reduced in the transformants overexpressing glutathione synthetase. Overexpression of bacterial γ-glutamylcysteinyl synthetase in poplar elevated levels of glutathione in the roots whereas overexpression of glutathione synthetase did not change glutathione levels (Rennenberg and Will 2000). Exposure to 5000 μM Cd over 5 days resulted in a loss of glutathione in all poplar lines and accumulation of more phytochelatins n=2, 3 and 4, particularly in the transformants overexpressing γ-

glutamylcysteinyl synthetase. The accumulation of phytochelatins in the roots did not avert damage to the leaves during the acute exposure to Cd.

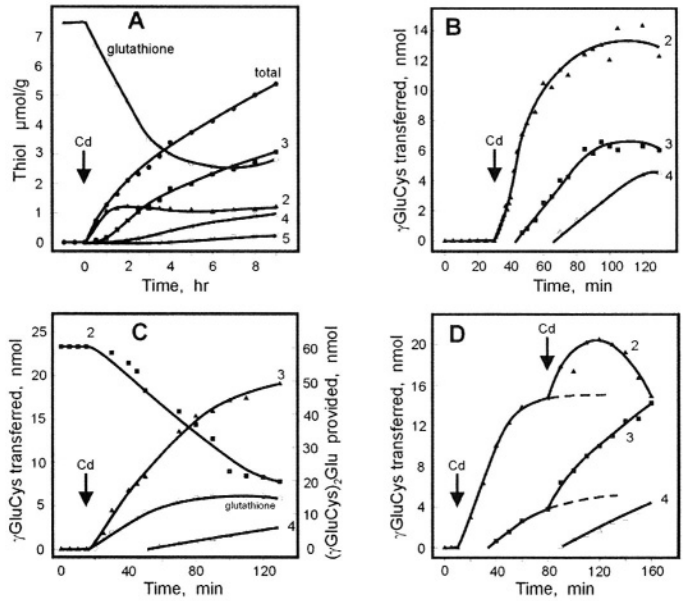

Figure 2. Time courses of phytochelatin appearance on adding Cd to cultured cells and to phytochelatin synthase *in vitro*. The digit associated with a response curve is the number of γGluCys units per phytochelatin molecule; n = 2, 3, 4 or 5. In **A** the contents of glutathione, individual phytochelatins and their total are per gram fresh mass of cultured cells of *Rauvolfia serpentina* (redrawn from Grill et al. 1987). In **B** 15-fold purified phytochelatin synthase from *Silene cucubalus* was incubated with glutathione, in **C** with $(\gamma GluCys)_2Gly$ in the absence of glutathione (redrawn from Grill et al. 1989). In **D** purified phytochelatin synthase from *S. cucubalus* was incubated with glutathione and a limited quantity of Cd followed by a second addition later (redrawn from Loeffler et al. 1989).

Glutathione as a substrate for phytochelatin synthase

An enzyme catalysing the transfer of the γGluCys moiety from glutathione to an acceptor molecule was purified extensively from cells of *Silene cucubalus* (Grill et al. 1989, Loeffler et al. 1989). It was a constitutive enzyme, its contents did not change with cell exposure to Cd. With glutathione as the starting substrate the enzyme first produced phytochelatin n=2 on addition of Cd and after some lags the n=3 and 4 oligomers (Figure 2B). These kinetics mimicked the appearance of phytochelatins in *R. serpen-*

tina cells (Figure 2A). In the initial reaction a glutathione molecule was the source of the γGluCys moiety that was added to another glutathione molecule to form $(\gamma GluCys)_2Gly$ with the release of Gly. The $(\gamma GluCys)_2Gly$ could then accept another γGluCys moiety from glutathione to form the n=3 oligomer and Gly (Figure 2B). Figure 2C shows the situation in the initial absence of glutathione where $(\gamma GluCys)_2Gly$ is the source of a γGluCys moiety that is transferred to another $(\gamma GluCys)_2Gly$ molecule to form the n=3 oligomer with liberation of a glutathione. Over time the $(\gamma GluCys)_2Gly$ was consumed as the n=3 oligomer was synthesized. Grill et al. (1989) classified the enzyme as a γ-glutamylcysteine dipeptidyl transpeptidase and gave it the trivial name phytochelatin synthase. The general reaction catalysed follows the equation:

$$(\gamma GluCys)_nGly + (\gamma GluCys)_nGly \Rightarrow (\gamma GluCys)_{n+1}Gly + (\gamma GluCys)_{n-1}Gly$$

where n=1, 2, 3 or more. The enzyme is assumed to operate in the cytosol because it is readily solubilized and glutathione synthetase occurs there to produce glutathione.

Enzyme activity was dependent on the presence of free Cd. Reactions were started with Cd (Figure 2B, C, D) and they stopped when the available Cd was complexed by sufficient product at a Cd:Cys ratio of 1:2 (Figure 2B, D). Addition of further Cd (Figure 2D) restarted the enzyme. Phytochelatin synthesis could also be stopped by adding EDTA or metal-free phytochelatin to complex free Cd (Loeffler et al. 1989). The activity of phytochelatin synthase is apparently self regulated by free Cd. The enzyme from *S. cucubalus* had a K_m for glutathione of 6.7 mM in the presence of 100 μM Cd at pH 7.9 and 35° C. From optimum concentrations of enzyme activation the order of efficacy was Cd>Ag> Pb> Cu> Hg> Zn> Sn> Au> As> In> Tl> Ge> Bi> Ga (Zenk 1996). These were among the same metals that caused phytochelatin appearance in plant cells (Table 2). In accord with an earlier study with cells, phytochelatin synthase was not activated by Al, Ca, Fe, Mg, Mn, Na or K.

Chen et al. (1997) used cultured tomato cells to isolate phytochelatin synthase. The enzyme purified by ammonium sulphate precipitation was used to corroborate the sequential production of enlarging phytochelatins (as in Figure 2B), that $(\gamma GluCys)_2Gly$ could serve as donor and acceptor of the γGluCys moiety (as in Figure 2C), and that certain metals were essential for catalysis. The tomato enzyme had a K_m of 7.7 mM for glutathione in the presence of 500 μM Cd at pH 8.0 and 35° C. From various concentrations of individual metals their order of effectiveness in catalysis was: Cd> Ag> Cu> Au> Zn> Fe> Hg = Pb. No activation was found with Ba, Ca, Co, K, Mg,

Mn, Mo, Na nor Ni. The enzyme was constitutive. A relatively crude preparation of phytochelatin synthase from cultured tobacco cells was optimally activated in the presence of 20 mM glutathione by Ag followed by Cd, Cu, Hg, Zn, Pb, Ni, Mn and Co (Nakazawa and Takenaga 1998). In the presence of 0.25 μM Cd, a concentration that only partly activated the enzyme, addition of 200-fold excess Zn led to further activation, as if Cd and Zn were interacting competitively. In the same circumstance, Pb, Ni, Mn and Co had neither additive nor synergistic effects.

Three groups have independently identified the gene for phytochelatin synthase. Ha et al. (1999) used a positional cloning strategy to show that *CAD1* in *A. thaliana* (Howden et al. 1995a) encoded phytochelatin synthase. This plant gene had 40 to 50 % homology with a sequence from the fission yeast *S. pombe* and the nematode *Caenorhabditis elegans.* The open reading frame encoded a 55 kDa protein of 485 amino acids, 10 of which were Cys in the carboxy-terminal half. Vatamaniuk et al. (1999) used suppression cloning to isolate a cDNA (*AtPCS1*) from *A. thaliana* that through heterologous expression in *Saccharomyces cerevisiae* conferred Cd tolerance through the accumulation of phytochelatin-based Cd-binding complexes. The purified AtPCS1 protein fused to a FLAG epitope was in the soluble fraction of transformed yeast cells as a single M_r 58,000 species. The highly purified recombinant AtPCS1-FLAG protein incorporated γGluCys from glutathione into $(\gamma GluCys)_2Gly$ at a rate of 30-35 μmol/mg·min, a rate 10^3-fold faster than found previously (Grill et al. 1989). Detailed kinetic and radiometric analyses of phytochelatin synthesis with the highly purified enzyme (Mari et al. 2000) conclusively supported the dipeptidyl mechanism originally proposed by Grill et al. (1989). However, free Cd or Zn were not necessary for catalysis, rather the binding of metal thiolate (e.g. $Cd\text{-}(GSH)_2$ or $Zn\text{-}(GSH)_2$) was essential (Vatamaniuk et al. 2000). The sufficiency of *S*-alkylglutathiones as both high affinity γGluCys acceptors and low affinity donors reflected the need for glutathione-like peptides containing blocked thiol groups for enzyme activity. A third group isolated *TaPCS1* from a screen of wheat genes involved in Cd resistance (Clemens et al. 1999). The open reading frame for this phytochelatin synthase predicted a polypeptide of 55 kDa, its expression in *S. cerevisiae* produced phytochelatins n=2 and 3. Both *AtPCS1*- and TaPCS1-mediated Cd tolerance was dependent on glutathione.

Does phytochelatin synthase produce all known families of γGluCys peptides? A start to this question was made with ammonium sulphate precipitated enzyme from roots of peas exposed to Cd (Klapheck et al. 1995). Peas have the monothiols γGluCys, glutathione and homoglutathione. The monothiol γGluCys was not a suitable substrate for phytochelatin synthase, an effect also observed with the tomato enzyme in the presence or absence of

glycine (Chen et al. 1997). With only the glutathione as substrate the pea enzyme synthesized $(\gamma GluCys)_2Gly$, but little of the n=2 counterpart peptides were formed when homoglutathione or γGluCysSer were the sources of the γGluCys moiety. In assays starting with 2.5 mM glutathione, increasing quantities of $(\gamma GluCys)_2\beta Ala$ appeared as concentrations of homoglutathione were raised, phytochelatin n=2 production diminished at the same time. The same effect occurred when γGluCysSer was provided along with glutathione. It was postulated that the enzyme had two binding sites, one for a γGluCys donor and another for a γGluCys acceptor. The donor-binding site had a high specificity for glutathione whereas the acceptor binding site had a low specificity for glutathione, homoglutathione and γGluCysSer. The kinetics observed suggested a competition by homoglutathione and γGluCysSer with glutathione for the γGluCys acceptor site (Klapheck et al. 1995). Further enzymatic work with other systems is required.

Other pathways for biosynthesis of γGluCys peptides

Ammonium sulphate precipitated crude enzyme from fission yeast (*S. pombe*) was used to follow incorporation of ^{35}S-labelled glutathione into γGluCys peptides (Hayashi et al. 1991b). The *in vitro* sequential synthesis of phytochelatin n=2, 3 and 4 was like the model for phytochelatin synthase. Cadmium was not essential for the reactions, perhaps sufficient Cu and Zn were present in the preparation to activate the enzyme. When the available glutathione was reduced from millimolar to low micromolar concentrations, dipeptidyl transfer caused formation of n=2, 3 and 4 oligomers of $(\gamma GluCys)_n$. This is the only *in vitro* evidence of a biosynthetic origin of the $(\gamma GluCys)_n$ family of peptides. In this alternative pathway later addition of glycine and partially purified glutathione synthetase caused conversion of $(\gamma GluCys)_n$ oligomers into the respective $(\gamma GluCys)_nGly$ oligomers. Phytochelatin synthase enzyme from peas and tomato did not use γGluCys to produce $(\gamma GluCys)_n$ (Klapheck et al. 1995, Chen et al. 1997).

A Cd-sensitive mutant of *S. pombe* with severely impaired phytochelatin synthesis and only 44 % of the glutathione content of wild type cells was the source of a DNA fragment involved with phytochelatin synthesis (Al-Lahham et al. 1999). Sequence analysis showed that the DNA encoded glutathione synthetase (GSH2, E.C. 6.3.2.3) with the mutant allele having a single base-pair exchange near the 3' end of the reading frame that changed a glycine to aspartate. It was deduced that the GSH2 gene encoded a bifunctional enzyme able to synthesize both glutathione and phytochelatins. Com-

plementation of the mutant with GSH2 from *A. thaliana* led to a partial restoration of phytochelatin synthesis in the mutant fission yeast (Leuchter et al. 1998). Whether the putative bifunctional activity of glutathione synthetase is a special case for fission yeast or also applies to plants requires further investigation.

FUNCTION OF GLUTATHIONE AND POLYTHIOL PEPTIDES

The monothiols and polythiols present in plants (Table 1) are biological ligands that provide S atoms for binding class B and borderline elements (Nieboer and Richardson 1980). This function may lower the concentrations of free metal ions in the cytosol to enable optimal metabolism. Excess metals in the environment present a particular stress on plants. Effective intracellular metal sequestration is part of the mechanism for survival.

Metal homeostasis

One function of the usually abundant glutathione in cells can be its action as an initial ligand for metals. In hepatoma cells Cu-glutathione complex served to bind the metal initially while later on class I metallothionein sequestered the Cu (Freedman et al. 1989). Metal-glutathione complexes have not been demonstrated *in planta.* Perhaps the rapidly responding phytochelatin synthase activity reduces the need for complexes with glutathione. From *in vitro* experiments it is clear that Cu, Pb and Hg transfer from metal-glutathione complex to phytochelatins n=2, 3 and 4 (Mehra and Mulchandani 1995, Mehra et al. 1995, 1996).

During subculturing of plant cells transfer into fresh medium provides abundant micronutrients including Cu and Zn. If these are absorbed rapidly there may be an internal oversupply until new Cu- and Zn-requiring apoenzymes are formed. Temporary sequestration through metal-induced phytochelatins may reduce the amount of free metal inside cells allowing undisturbed metabolism. Such homeostasis was apparent for *R. serpentina* cells (Figure 3A, Grill et al. 1988). The cells were initially low in phytochelatins because Cu and Zn were omitted from the medium in the prior culture. During logarithmic growth, Cu and Zn disappeared from the medium and phytochelatins accumulated. Once the medium was depleted of Cu and most Zn, phytochelatins began to disappear with a half-time of about 2.5 days as the

cells metabolized in stationary phase. It is assumed that phytochelatins bound Cu and Zn, no direct assessments of their complexes are available.

A variety of enzymes are activated by metals. The transfer of metal from a temporary store such as phytochelatins to newly synthesized apo forms of metal-requiring enzymes is part of the homeostasis suggested in Figure 3A.

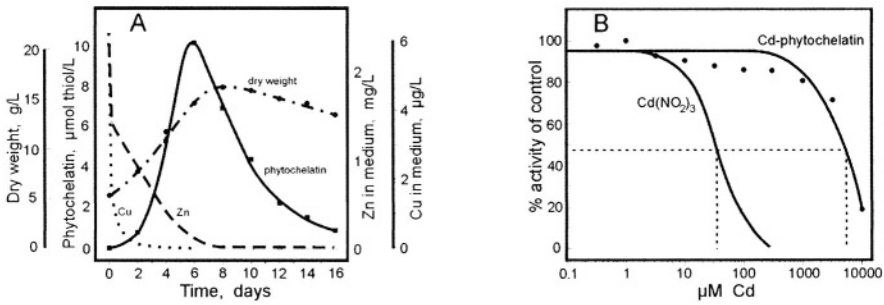

Figure 3. **A** Time course for growth and total phytochelatin content in *Rauvolfia serpentina* cells transferred to fresh complete culture medium. The Cu and Zn in the medium were monitored along with phytochelatin content in the multiplying cells (redrawn from Grill et al. 1988). **B** Effect of increasing Cd salt and Cd-phytochelatin complex isolated from *Rauvolfia serpentina* on the *in vitro* activity of glyceraldehyde-3-phosphate dehydrogenase from *Silene cucubalus* suspension cultures (redrawn from Kneer and Zenk 1992).

The apo form of diamino oxidase from pea was reactivated *in vitro* by Cu-phytochelatin n=2, or the plant mixture with predominantly n=2 and 3, nearly equally as well as with the same concentration of inorganic Cu (Thumann et al. 1991). However, Cu complexed with phytochelatins n=3 and 4 was less efficient. The apo form of Zn-requiring bovine carbonic anhydrase was activated by Zn-phytochelatin n=2 complex nearly as well as with the same concentration of inorganic Zn. The phytochelatin n=7 complex with Zn was much less effective. The lesser reactivations by Cu and Zn in complexes with larger oligomers may reflect a more efficient binding of metal through longer groups of cysteines. The counterpart action, that of protecting plant enzymes from metal poisoning, was examined by Kneer and Zenk (1992). The activity of glyceraldehyde-3-phosphate dehydrogenase from *S. cucubalus* suspension cultures was protected from Cd inhibition more effectively when Cd was added as Cd-phytochelatin complex rather than inorganic Cd (Figure 3B). Similar patterns were demonstrated for the enzymes alcohol dehydrogenase and nitrate reductase from *Nicotiana plumbaginifolia,* ribulose-l,5-bisphophate carboxylase oxygenase (Rubisco) from spinach and urease from jackbeans. Rubisco, which is more sensitive of Zn than Cd, was

effectively protected in the presence of Zn-phytochelatin complex. The activity of nitrate reductase poisoned by Cd-acetate was fully restored when apo phytochelatin n=3 was added. Glutathione was at least 1000-fold less effective than phytochelatin in attenuating the inhibition by Cd, citrate was ineffective. Such *in vitro* experiments support the premise that phytochelatins participate in metal homeostasis. The cad1-3 mutant of *A. thaliana* has no detectable γGluCys peptides nor phytochelatin synthase activity, yet growth is normal in the presence of micronutrient concentrations of Cu and Zn (Howden et al. 1995b). Whether the variety of γGluCys peptides described in Table 1 show similar effects of metal transfer or prevention of metal poisoning by an inducing metal remains unsettled.

Sequestering cadmium in complexes

The ability of γGluCys peptides to bind certain metals has been characterized extensively for Cd. Extracts are prepared for gel filtration chromatography using alkaline buffers that maintain Cd-peptide interactions. General protocols have been described (Grill et al. 1991, Hayashi et al. 199la, Mutoh and Hayashi 1991, Rauser 1991) and current publications show modifications in buffers and gel filtration media. In many cases most of the Cd applied to the gel filtration column appears as two major Cd-binding complexes shown as regions II and III in Figure 4. The Cd at the void volume (region I) may be considered non-specifically bound Cd. Cadmium eluting in the position of free inorganic Cd (fractions 80-110, Figure 4) is usually absent. Regions II and III are named the high molecular weight (HMW) and low molecular weight (LMW) complexes, respectively. They are induced in cells and tissues exposed to Cd. The separations achieved through gel filtration are influenced by a number of factors. Concentrating Cd-binding complexes through anion exchange chromatography in preparation for gel filtration (Rauser 1991, Rauser and Meuwly 1995) seems to exclude the LMW complex from the preparation as if the anionic charge density in this complex is less than in the HMW form. The extracts used to develop Figure 4 were concentrated by lyophilization. The distribution of Cd between high and low molecular weight complexes varies between species and with Cd dose. For maize cultivar 2497 (Figure 4) the HMW complex accounted for 1.55-times the Cd present in the LMW complex. For maize cultivar 37701 under the same conditions, Cd as HMW complex exceeded that in the LMW form by 2.3 to 3.5-times (Rauser 2000). Early in the exposure of *S. pombe* cells to Cd the HMW and LMW complexes accounted for about the same amount of Cd and the complexes were resolved completely (Murasugi et al.

1981). At a later exposure the HMW complex dominated and the LMW complex now chromatographed as a trailing shoulder on a large peak. By decreasing the flow rate, size of fractions and re-chromatography of pooled fractions, the major Cd-binding complex found for *R. serpentina* cells (Grill et al. 1985) could be separated into HMW, medium molecular weight and LMW complexes (Kneer and Zenk 1997). Rather than being discrete homogeneous entities, these complexes are themselves best viewed as mixed complexes. A variety of γGluCys peptides combine with Cd and acid-labile sulphide to make up the complexes (Table 3). Assuming that one molecule of the least abundant γGluCys peptide occurred in a complex and then calculating the others in proportion to their abundance, gives theoretical masses far in excess of those suggested by gel filtration. Apparent molecular weights for the HMW complex from *R. serpentina* range from 8000 at low ionic strength to 3600 at ionic strengths at or above 300 mM (Grill et al. 1987). The HMW and LMW complexes from fission yeast showed apparent molecular weights of 4000 and 1800, respectively (Murasugi et al. 1981). No molecular weight data are available with methods other than gel filtration. Isolation of only a HMW complex from *S. cucubalus* cells remains unexplained (Table 3).

The Cd-binding complexes isolated from yeasts and plants have been characterized in a variety of ways. The HMW complex is documented most extensively because of its abundance. Ultraviolet absorption spectra of Cd-binding complexes show a shoulder of absorption at 254-257 nm, typical of Cd-thiolate bonding. This is the reason that A_{254} was followed in gel filtrations (Figure 4). Clearer and distinctive evidence for Cd-thiol coordination was found through measurements of circular dichroism in the 200 to 320 nm range (Rauser et al. 1983, Grill et al. 1985). Amino acid analyses of purified complexes have been superseded by analyses of the γGluCys peptides present (e.g. Table 3). The complexes are acidified to liberate the apo peptides for separation by HPLC. During acidification there may be noticeable release of hydrogen sulphide indicating the presence of sulphide in the complex. Such acid-labile sulphide is usually determined by the methylene blue method of King and Morris (1967). Acid-labile sulphide, when it has been measured, is a constant feature of HMW complexes from a variety of sources yet it is low or absent in many LMW complexes (Table 3, Murasugi et al. 1983, Speiser et al. 1992). The low and high molecular Cd-binding complexes from maize roots are unusual in that the sulphide to peptide thiol ratio is relatively constant, whereas it is lower for the LMW complexes from other sources (Table 3, Rauser 2000).

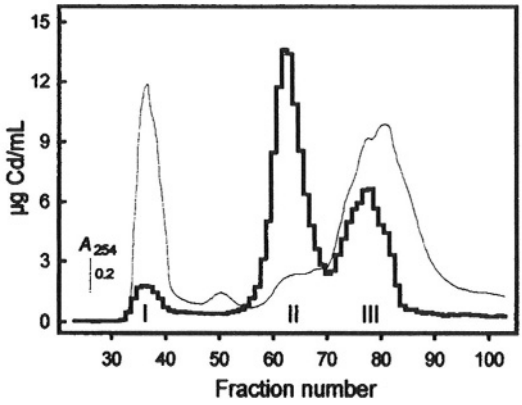

Figure 4. Gel filtration chromatography of an extract from maize roots exposed to 3 µM Cd for 5 days. Six sequential extracts of maize roots (Cargill hybrid 2497) with Hepes (pH 8.6) buffer were concentrated by lyophilization. Material equivalent to 5.62 g fresh mass was applied to a 2.6 i.d. x 41.2 cm column of Sephacryl S-100. The column was operated with N_2-purged 10 mM Hepes (pH 8.0) and 300 mM KCl at a flow rate of 0.8 mL/min and 4 °C. The histogram shows the concentration of Cd in the fractions (2.4 to 2.5 mL), the continuous trace indicates the A_{254}. Regions II and III were pooled for peptide and sulphide analyses (redrawn from Rauser 2000).

Since Cd-binding complexes have not yet been crystallized, measurements of extended X-ray fine structure (EXAFS) have been used to assess the chemical environment of Cd. The first study was for a low sulphide HMW complex from *R. serpentina* (Strasdeit et al. 1991). One atom of Cd interacted with four sulphurs each provided by a cysteine to form $Cd(SCys)_4$ centres. The Cd-S bond distance was 0.252 ± 0.002 nm. The carboxylate groups, especially from the abundant glutamic acid residues in the γGluCys peptides, were not coordinating Cd. Their location towards the surface accounted for the high negative charge associated with Cd-binding complexes. Examination of a sulphide-rich HMW complex from maize by EXAFS also indicated a predominantly tetrahedral coordination of Cd by sulphur at a bond distance of 0.254 nm (Pickering et al. 1999). Well-defined Cd-Cd interactions in a polynuclear manner were not evident because of high static disorder, however, this did not mean that isolated Cd-tetrathiolate ligation occurred exclusively. The sulphur K-edge showed characteristics consistent with sulphide bound in a cluster with Cd, parallelling that in the crystallographically defined Cd-thiophenolate ion $[S_4Cd_{10}(SPh)_{16}]^{4-}$. This feature strongly suggested a polynuclear Cd cluster with sulphide in the HMW complex examined.

Table 3. Composition of isolated Cd-binding complexes from plants. The data for *Rauvolfia serpentina* and *Silene cucubalus* are the contents per mg of isolated complex (Kneer and Zenk 1997). For maize the contents are for HPLC separations of the material remaining after Cd analysis in regions II and III of Figure 4. Selected ratios are provided.

	R. serpentina			*S. cucubalus*	maize (Figure 4)	
	LMW	MMW	HMW	HMW	LMW	HMW
Form of SH			[nmol SH]			
glutathione + γGluCys					5.41	3.99
$(\gamma GluCys)_2Gly$	42	19	14	14	0.99	1.80
$(\gamma GluCys)_3Gly$	779	85	246	1069	11.56	43.73
$(\gamma GluCys)_4Gly$	71	1064	475	492	6.61	45.37
$(\gamma GluCys)_5Gly$	0	317	81	24	5.45	18.83
$(\gamma GluCys)_2$	0	238	58	0	4.00	6.30
$(\gamma GluCys)_3$	0	108	27	0	24.79	76.20
$(\gamma GluCys)_4$					13.24	52.95
$(\gamma GluCys)_5$					2.68	17.16
γGluCysGlu					0.90	1.11
$(\gamma GluCys)_2Glu$					1.17	1.55
$(\gamma GluCys)_3Glu$					6.61	25.54
$(\gamma GluCys)_4Glu$					1.36	4.03
$(\gamma GluCys)_5Glu$					3.54	9.08
Total SH	892	1832	900	1598	88.33	308.37
nmol S^{2-}	10	18	255	710	29.34	104.65
nmol Cd	302	786	624	1574	65.12	529.87
			selected ratios			
S^{2-} : Cd ratio	0.03:1	0.02:1	0.41:1	0.45:1	0.45:1	0.20:1
S^{2-} : peptide SH ratio	0.01:1	0.01:1	0.28:1	0.44:1	0.33:1	0.34:1
Cd: peptide SH ratio	0.34:1	0.43:1	0.69:1	0.98:1	0.74:1	1.72:1
Cd: peptide SH+S^{2-} ratio	0.34:1	0.42:1	0.54:1	0.68:1	0.55:1	1.28:1

LMW, low molecular weight; MMW, medium molecular weight, HMW, high molecular weight.

The HMW complexes from *S. pombe* and *C. glabrata* cultured on high Cd concentrations appeared as dense aggregates of 2 nm diameter particles (Dameron et al. 1989). The X-ray diffraction patterns indicated the presence of a CdS crystallite. It was estimated that about 80 CdS units were coated by 30 molecules of glutathione, $(\gamma GluCys)_2Gly$ and $(\gamma GluCys)_2$. Formation of such crystallites is an example of biomineralization where biopolymers (i.e. the γGluCys peptides) provide an ordered structure to one component (i.e. Cd) so that another constituent (i.e. sulphide) can be added to generate a specific crystal. The sulphide-rich portions of Cd-binding complex from tomato roots exposed to 100 μM Cd had optical properties typical of small

GdS crystallites (Reese et al. 1992). The sulphide to Cd ratio was around 0.4:1. Similar ratios are known for *R. serpentina* and *S. cucubalus* complexes (Table 3), presence of CdS crystallites was not evaluated.

Model of cellular location

The LMW and HMW Cd-binding complexes are buffer soluble as if they are in easily accessible compartments in mature cells. Mutants of *S. pombe* and *A. thaliana* unable to produce LMW and HMW Cd-binding complexes are Cd-hypersensitive or particularly vulnerable to Cd poisoning (Mutoh and Hayashi 1988, Howden et al. 1995a). Mutants able to form LMW complex only could deal with some Cd but were more sensitive than those producing both LMW and HMW complexes. The partial effectiveness of the LMW complex is related to its lesser capacity for Cd per mole of thiol or thiol plus sulphide compared to the HMW complex (Table 3). The gene *hmt1* was found to complement a mutant of *S. pombe* that was able to produce phytochelatins yet not form HMW complex. The *hmt1* gene shared sequence identity with the superfamily of ATP-binding cassette-type transport proteins (Ortiz et al. 1992). The native HMT1 polypeptide was located in the vacuolar membrane (Ortiz et al. 1995). The protein transported apophytochelatins into vacuolar vesicles in an ATP-dependent manner. The electrochemical potential gradient generated by the vacuolar-type ATPase could not drive the peptide transport. Sulphide-poor LMW complex was transported more efficiently than the sulphide-rich HMW complex. The HMT1 polypeptide did not transport Cd^{2+}. The model shown in Figure 5 incorporates what these workers envisaged along with other information. In the cytosol glutathione is used to produce phytochelatins that bind Cd and aggregate into the LMW Cd-binding complex. The LMW complex is moved into the vacuole by the ATP-binding cassette-type transporter at the direct expense of ATP. Once inside the vacuole, more Cd, transported by a Cd^{2+}/H^{+} antiporter, and sulphide are added to produce the HMW complex. In this model the LMW complex functions as a cytosolic carrier and the vacuolar HMW complex is the major storage form of cellular Cd. In yeast the source of sulphide for the HMW complex is attributed to purine metabolism (Juang et al. 1993).

The model in Figure 5 is supported by data from plants in several ways. Tonoplast vesicles from oat roots show MgATP-dependent transport of phytochelatins and of Cd-phytochelatin complex (Salt and Rauser 1995). Several properties of this activity corresponded to those of the superfamily of ATP-binding cassette-type transporters. Phytochelatin transport was not driven by the electrochemical potential gradient generated by the vacuolar-type ATPase. Oat root tonoplast vesicles also have a Cd^{2+}/H^{+} antiporter (Salt

and Wagner 1993). The vacuolar-type ATPase generating a proton gradient is well recognized in plants. Addition of Cd to newly arrived LMW complex in the vacuole to form HMW complex is evident from the higher ratios of Cd:peptide thiol or to peptide thiol plus sulphide shown for two plants in Table 3. Direct evidence for the subcellular localization of LMW and HMW complexes in the cytosol and the vacuole, respectively, is lacking. Indirect, partial evidence comes from mesophyll protoplasts of tobacco exposed to Cd and the vacuoles derived from those protoplasts. The vacuoles contained all the Cd and phytochelatins found in the protoplasts (Vögeli-Lange and Wagner 1990). Cadmium-binding complexes per se were not determined.

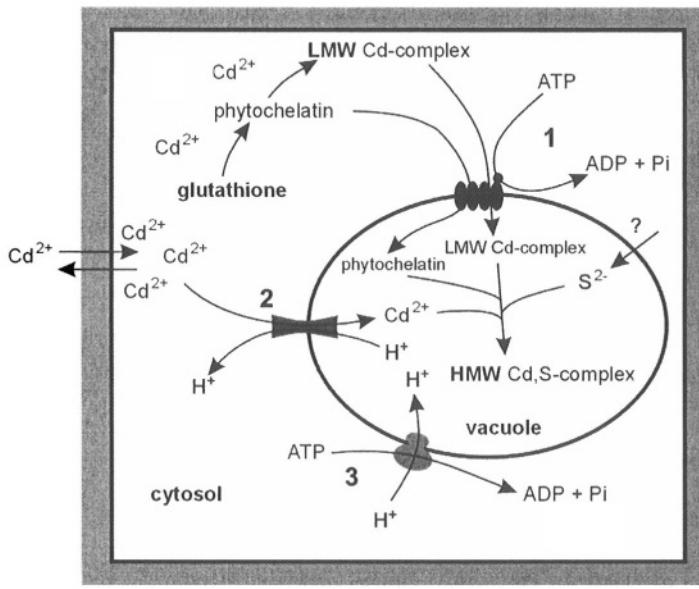

Figure 5. A model of the intracellular location of low and high molecular weight Cd-binding complexes derived from glutathione and associated transport mechanisms. The model is adapted from Ortiz et al. (1995) and is augmented with the observed transport of apophytochelatin. The established transport activities across the tonop*last are: 1, the ATP-binding cassette-type transporter (Ortiz et al. 1995, Salt and Rauser 1995); 2, the Cd^{2+}/H^{+} antiporter (Salt and Wagner 1993); and 3, the vacuolar-type ATPase generating a proton gradient. Mechanisms for transport of sulphide into the vacuole and of Cd across the plasma membrane are unclear.

Figure 5 shows an alternate route where phytochelatins formed in the cytosol are transported into the vacuole as apopeptides (Ortiz et al. 1995, Salt and Rauser 1995) where they combine with Cd and sulphide to form HMW

complex. How the γGluCys peptides other than phytochelatins are mobilized within the cells of plants where they are found remains to be explored. Do the other γGluCys peptides follow the same pathway as phytochelatins? The constancy of sulphide to peptide thiol in LMW and HMW complexes from maize (Table 3) demands addition of sulphide in the cytosol rather than in the vacuole. How prevalent is this situation? Transport of Cd^{2+} and related metals at the plasma membrane is a developing research area.

Sequestering other metals in complexes

The interactions in plants between glutathione, γGluCys peptides and metals other than Cd have received little scrutiny. It must be recognized that of the many metals which induce the appearance of γGluCys peptides (Table 2) the majority do so to limited extents. As a consequence the putative metal-peptide complexes may be in low abundance.

Continuous introduction of effluent from an analytical gel filtration column into an inductively coupled plasma-atomic emission spectrometer allowed simultaneous assessment of various elements (Kubota et al. 1995). For the Cd-exposed roots of *Rubia tinctorum* a Cd peak was found to contain sulphur, suggestive of the γGluCys peptides they found by other means. For Ag-treated roots a single Ag peak was found with some Cu coincident, however, a Zn peak eluted later (Maitani et al. 1996). The large and small peaks of Cd-binding material also contained some Cu. In Cu-treated roots a large and small peak bore Cu. Complexes binding Cu, Pb or Zn obtained from *R. serpentina* were based on phytochelatins (Grill 1989), however, the Zn was labile leading to substantial loss of Zn during purification and a low Zn:sulphur ratio. In alkaline extracts of the aquatic macrophytes *Hydrilla verticillata* and *Vallisneria spiralis* Hg appeared in three major peaks that were accompanied by non-protein thiol (Gupta et al. 1998). Phytochelatins n=2 and 3 were detected in acid extracts of the Hg-exposed plants.

Involvement in metal tolerance

The use of buthionine sulphoximine to lower glutathione pools and mutants deficient in glutathione biosynthesis or of γGluCys peptides show that these thiol compounds are essential for plants to deal with metals given in various doses. The term metal tolerance is often used in this context, however, it is used in different ways by various workers. Ecotypes of various

plants are known to thrive over a large range of external metal concentrations. Such ecotypes are said to be metal tolerant in comparison to those ecotypes that thrive within a narrow range of metal concentrations. Differential metal tolerance is displayed by these ecotypes. In *Holcus lanatus, Agrostis capillaris*, *Chlamydomonas reinhardtii*, *Mimulus guttatus* and *Silene vulgaris* (=*cucubalus*) one or two major genes give differential tolerance of As, Cd or Cu with other genes as modifiers (Macnair 1993). No locus of differential metal tolerance has yet been isolated and cloned for a plant.

Since γGluCys peptides are metal inducible, and bind metal, these molecules are considered as candidates in mechanisms of differential metal tolerance. The simplest expectation is that the metal tolerant ecotypes produce more metal-binding γGluCys peptides than do the non-tolerant ecotypes. This expectation should apply to root apices since differential metal tolerance is identified through root growth assays that are expressions of metabolism in the apices. The expectation was not met for Cu- and Cd-tolerant ecotypes of *Silene vulgaris* (=*cucubalus*) (De Knecht et al. 1994 and references therein). The root apices of metal tolerant ecotypes contained lower or the same amounts of non-protein thiols as did the non-tolerant ecotypes at external metal concentrations that caused the equivalent root growth inhibition. The non-protein thiols measured included γGluCys peptides with glutathione excluded. The Cd-binding complexes based on γGluCys peptides, particularly in the whole root system (Figure 4), were seen as sinks for excess Cd rather than the cause of differential tolerance of Cd. The correlation between the degree of Cu tolerance in root systems of *A. thaliana* and non-protein thiols was less strong than with the quantity of two mRNAs of class II metallothioneins (Murphy and Taiz 1995). Metal-binding complexes and their composition remain to be documented for root apices. Progress in defining the role of glutathione and of γGluCys peptides in metal tolerance requires simultaneous measurement in root apices of complexes based on these ligands as well as the class II metallothioneins and perhaps organic acids and phytin.

EPILOGUE

A reasonably large and diverse data set is available on how plants use glutathione in reaction to Cd. The fact that other metals act like Cd to induce the appearance of γGluCys peptides does not necessarily mean that formation of complexes and their subcellular apportionment is as for Cd. More specific data on these points are required for metals such as Cu, Hg, Pb and Zn. Plant cell suspension cultures are an important source of experimental

data, however, they cannot express in entirety what happens in an organized tissue such as a root attached to a shoot. Some plant responses to excess metals, such as differential metal tolerance expressed in roots, rely on the metabolism in specific, small regions of the plant. The reactions in root apices are masked when the entire root system is analyzed. There is a need to miniaturize the analytical procedures described in this chapter or to devise new ones to accommodate such tissue specific questions.

The concentrations of Cd used in the experiments described vary tremendously, ranging from 3 μM for maize (e.g. Figure 4, Rauser and Meuwly 1995) through 1000 μM for yeasts to produce CdS crystallites (Dameron et al. 1989) to 5000 μM for transgenic poplars under chronic acute contamination (Rennenberg and Will 2000). Soils used for agricultural production contain low levels of Cd estimated at 0.04 to 0.32 μM in soil solution (Wagner 1993). In highly polluted soil the concentrations may rise 10-fold. Some caution may be appropriate before invoking the responses described in this chapter as operative to plants in polluted soils. Work at lower concentrations of metals, and even combinations of metals, that better reflect polluted soils is necessary, not only for Cd, but for the other metals that dramatically influence the utilization of glutathione in plants.

Manipulation of plants through genetic engineering to alter γ-glutamylcysteine and glutathione contents is at hand (see Foyer and Noctor in this volume). Identification of the gene for phytochelatin synthase now makes direct targeting of γGluCys peptides possible. The fact that Cd-binding complexes based on γGluCys peptides sequester high proportions of metal in mature cells (e.g. 76 to 85 % of the Cd in roots of maize seedlings; Rauser 2000) can be viewed as complementary, rather than exclusionary, to other ligands such as class II metallothioneins that may operate in embryonic regions of plants and in leaves.

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Chapter 7

THE ROLE OF GLUTATHIONE AND GLUTATHIONE *S*-TRANSFERASES IN PLANT REACTION AND ADAPTATION TO XENOBIOTICS

Peter Schröder

INTRODUCTION

All organisms are frequently exposed to an array of potentially toxic substances, be they inorganic or organic in chemical nature. These substances may originate naturally from fires, volcano eruptions or processes of biodegradation. They may as well spring from microbial or animal metabolism, and also from the plant's secondary metabolism (Naumann 1993, Altshuller 1983). They may act as active substances in defence or in allelopathic reactions. Furthermore, increasing industrialization has provided two novel sources of foreign compounds: first by the invention and use of agrochemicals for the protection of crops and the control of pests and weeds, and second by the emission of organic xenobiotics in the process of chemical production of goods or the use of synthetic chemicals. The latter compounds of solely anthropogenic origin represent a threat to our environment as they are emitted without any control. For plants, the situation is especially awkward as they root in the ground and are bound to survive on a certain site. Hence they have to rely on effective detoxification mechanisms in a special manner.

The uptake of xenobiotics from whatever polluted medium, i.e. air, water or soil, follows the laws of phase distribution and diffusion and hence plants have only limited possibilities to avoid accumulation of foreign compounds in their tissue and the connected detrimental consequences. In the context of glutathione mediated detoxification processes, especially those foreign compounds bearing electrophilic sites are of major importance.

D. Grill et al. (eds.), Significance of Glutathione to Plant Adaptation to the Environment, 155-183.

DETOXIFICATION OF ELECTROPHILIC XENOBIOTICS

Various electrophilic xenobiotics, i.e. compounds with centres of low electron density that can accept an electron pair to form a covalent bond, exhibit the tendency to react spontaneously with nucleophilic sites (i.e. centres of electron richness, non-bonded pairs of electrons or π bonds) of biomolecules. Thus, electrophilic xenobiotics may be highly dangerous to the cell, because they are able to bind to proteins and genetic material, i.e. DNA and RNA and thereby disturb metabolic networks.

The action of electrophilic xenobiotics seems to be dependent on their cellular counterparts. There is a preference for the reactions between xenobiotics and biomolecular partners, which may be explained by the concept of hard and soft nucleophiles/electrophiles (Coleman et al. 1997). In this concept, "hard" and "soft" are attributes of chemical bonds or functions describing the reactivity of the nucleophilic/electrophilic centres. Hence a high degree of polarization with positive or negative charges at the reactive centres characterizes "hard" nucleophiles/electrophiles, whereas "softness" is connected to polarization by low positive or negative charges at reactive sites. The concept is based on the observation that electrophiles and nucleophiles of similar hardness/softness react with each other (Table 1). Consequently, sulphur in peptides (i.e. glutathione) would be the softest nucleophilic site and be able to react with a large number of xenobiotics with corresponding properties.

Table 1. Reactivities between nucleophilic biomolecules and electrophilic xenobiotics (from Coleman et al. 1997, with modifications).

Nucleophilic site	*Softness/Hardness*	*Electrophilic site*
sulphur in cysteinyl residues of proteins or GSH	**Soft**	polarized double bonds, aldehydes
sulphur in methionyl residues of proteins		epoxides, alkyl sulphates, alkyl halides, stained ring lactones
amino groups in proteins (Arg, Lys, His)		
amino groups of purines in DNA and RNA		arylcarbonium ions
oxygens of purines and pyrimidines		benzylic carbonium and nitrenium ions
phosphate oxygen of RNA and DNA	**Hard**	alkylcarbonium ions

By this ability to spontaneously react with electrophiles, glutathione and its analogues have been suggested to possess a central role as cellular protectants against chemical polarization of soft sulphur containing residues in proteins by xenobiotics.

The mechanism of detoxification is conjugation between the xenobiotic at its electrophilic site and the thio-group of glutathione. This conjugation reaction will proceed spontaneously with a large number of electrophilic xenobiotics of similar softness. The reaction with hard electrophiles requires additional enzymatic support, which is provided by glutathione *S*-transferase isoenzymes. In any case detoxification totally depends on the availability of glutathione. The homeostasis of glutathione inside the plant is maintained by a complex regulation process (see other chapters of this volume) with synthesis, degradation and long range transport as visible end points. Perturbation of homeostasis and depletion of GSH pools may therefore lead to severe disturbance in a plant's detoxification capacity.

Prerequisites for glutathione dependent detoxification:
- electrophilic centres on the xenobiotic to be attacked
- adequate supply of glutathione or its analogues
- glutathione *S*- transferase with respective substrate specificity

Bearing two carboxylic acid groups, one amino group and one thiol group and having two peptide bonds, glutathione is highly hydrophilic. Glutathione conjugation of hydrophobic electrophilic compounds thus leads to a loss of their lipophilicity by converting the parent compound into an amphiphilic product with a bulk hydrophilic region and the non-polar hydrophobic region. This change of physico-chemical properties impedes the mobility of the foreign compound and inhibits further partitioning into membranes as well as the diffusion between compartments. Even more important, the products of this reaction are subject to ionisation at cellular pH and definitely restricted in their availability to the cells and tissues.

In consequence, as has been pointed out recently (Coleman et al. 1997), (a) the biological half-life of the xenobiotic in the cell is decreased, (b) the time the organism is exposed to the toxin is reduced and (c) an accumulation in critical tissues or compartments of the cells is avoided.

However, amphiphilic metabolites have been shown in the literature to act inhibitory on cytoplasmic enzyme action. This might be one of the reasons why the concentration of glutathione conjugates in the cytosol has to be kept low.

CLASSIFICATION OF DETOXIFICATION REACTIONS: 3-PHASE MODEL

Some xenobiotic compounds have to be activated for conjugation with glutathione or GST mediated conjugation. Most if not all conjugation products then undergo metabolism, transport and long term storage or excretion from the living cells in plants. The parallels with animal metabolism of xenobiotics in intestine, liver and kidney have been pointed out early by Richard Shimabukuro (1971) from the USDA Bioscience Research Laboratories in Fargo, ND. His ideas have been confirmed later by several authors (Lamoureux and Rusness 1989, Lamoureux et al. 1991, Coupland 1991, Coleman et al. 1997, Schröder 1997) and have led to the „green liver concept" favoured by Sandermann and co-workers (Sandermann 1994, Sandermann et al. 1997). All concepts have in common the option of xenobiotic activation by P450 monooxygenases and related enzymes in a first step designated phase I, the detoxification in the true meaning of the word in phase II and the metabolism, breakdown or final storage in phase III (Figure 1).

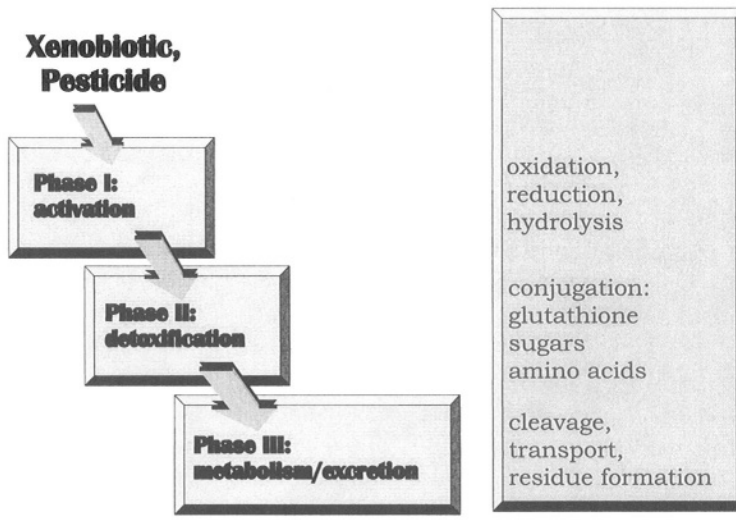

Figure 1. The three-phase model of detoxification in animals and plants (adapted from Shimabukuro 1976, Coupland 1991, Sandermann et al. 1997). In phase I xenobiotic molecules are activated via oxidation, reduction or hydrolysis, and in phase II detoxification is achieved by conjugating biomolecules, e.g. sugars, amino acids or glutathione to the activated sites. Compounds with sufficiently high electrophilicity may be conjugated without activation. Phase III is characterized as cleavage, secondary conjugation and metabolism of conjugates and may include compartimentation into the vacuole, the apoplast or the cell wall.

In addition to internal storage processes, excretion into the soil with root exudates or into the atmosphere after volatilization (Lamoureux et al. 1993) may be a significant phase III step in plants for some compounds as has recently been shown for metabolites of the diphenylether herbicide fluorodifen.

Of all phases, phase II has the largest effect on the effective toxicity of foreign compounds taken up by the cell. It has to be mentioned that for a large number of xenobiotics, especially after hydroxylation reactions in phase I, sugar conjugation by glycosyltransferase action is the detoxification step. This reaction is very effective, however it has frequently been observed to be reversible. Numerous glycosidases exist that may become active under certain conditions in the cell and liberate the hydroxylated parent xenobiotic by cleaving the glycosyl residue. A way to overcome this problem of retarded toxicity seems to be the formation of bound residues outside of the cytosol, in the cell wall.

For glutathione conjugation, the situation is different. The mechanism of glutathione binding to a xenobiotic includes the cleavage of reactive centres, mostly halogens, from the xenobiotic in exchange with the cysteinyl sulphur. Even if cleavage of this bond occurred (see below) the resulting product would be devoid of the electrophilic centre of the parent and would therefore not be as reactive as before. Furthermore, experimental evidence has shown that the sulphur bond to the molecule is very stable because cleavage of conjugates includes destruction of the cysteinyl moiety. Thus the former xenobiotic, even if liberated (by metabolic activity), will not have the chemical nor the toxicological characteristics of the original compound, i.e. the electrophilicity will be lowered. These considerations are valid for most of the glutathione dependent conjugation reactions reported, except for a special type of reactions on compounds with reactive carbon-carbon double bonds neighboured by an electron-withdrawing group (Talalay et al. 1988). The conjugation on these bonds is a so-called Michael reaction and leads to a labile conjugate that may be sensitive to pH changes (Ishikawa 1987).

GLUTATHIONE *S*-TRANSFERASES

Although numerous electrophilic compounds are detoxified by glutathione conjugation at appreciable rates, the presence of the enzyme, glutathione *S*-transferase (GST, E.C. 2.5.1.18) speeds up the reactions considerably. For most of the xenobiotics investigated, the role of the enzyme seems to be more to promote the proximity of the reactants than to catalyse the reaction itself.

Table 2. Significance of the formation of glutathione conjugates from herbicides and selected foreign compounds in plants. GS-: glutathione-; Cys: cysteine-; cysgly: cysteinylglycine-conjugate (from Schröder 1997, modified). References: a, Breaux et al. 1987; b, Frear et al. 1983; c, Wolf et al. 1996; d, Shimabukuro et al. 1971; e, v.d. Trenck and Sandermann 1978; f, Lamoureux and Rusness 1983; g, Rusness and Still, 1977; h, Sweetster et al. 1982; i, Brown and Neighbors 1978; k, Schröder 1996; l, Blattmann et al. 1986; m, Mayer et al. 1981; n, Riechers et al. 1996; o, Ezra and Stevenson 1985; p, Edwards and Cole 1996; q, Shimabukuro et al. 1973; r, Lamoureux and Rusness 1980; s, Schröder et al. 1990; t, Ezra and Stevenson 1986; u, Frear et al. 1985; v, Schröder 1993; w, Lamoureux et al. 1971; x, Edwards and Owen 1989; y, Lamoureux and Rusness 1986; z, Wittenbach et al. 1994.

Herbicide	plant species	conjugate described	Reference
Acetochlor		GS-, Cys	a
Acifluorfen	soybean	GS-, Cys, related	b
Alachlor	barley	GS-, Cys	c
Atrazine	corn	GS-, Cys, N-Cys	d
Benzo(a)pyren		GS-	e
Butachlor	peanut	GS-, Cys, malonylcys	f
Clorpropham	oats	GS-, Cys	g
Chlorimuron ethyl	soybean	GS-	h
Chlorsulphuron		GS-	i
Dichloromethane	spruce	GS-	k
Dimetachlor		GS-Cys, thiolactic acid	l
Dimethametryn		GS-, Cys, N-cys	m
Dimethenamid	wheat	GS-, γ-GC, Cysgly, Cys	n
EPTC	millet	GS-, malonylthiolactic acid	o
Fenoxaprop ethyl	wheat	GS-	p
Fluorodifen	pea,peanut, spruce	GS-, Cys, glucosides	q, r, s
Metazachlor	corn	GS-	t
Metolachlor		GS-, Cys, thiolactic acid	l
Metribuzin	tomato, soybean	GS-	u
Molinate	peanut	GS-, Cys, malonylcys	f, r
Pebulate		GS-	r
PCNB	onion, spruce	GS-, di-GS-, Cys, S-CH_3-cys	f,q
Perchloroethene	spruce	GS-	v
Pretilachlor		GS-, Cys, thiolactic acid	l
Propachlor	soybean, spruce	GS-, Cys, related	q, s, w
Terbutryne	potato, wheat	GS-	x
Tridiphane	giant foxtail, corn	GS-	y
Trisulphuron Methyl	sugarbeet	GS-, Cys, related	z
Trichlorethene	spruce	GS-	v

Glutathione *S*-transferases and glutathione seem to have co-evoluted jointly as they are found ubiquitously in animals, bacteria and plants. GSTs have first been described in animals in 1961 (Booth et al. 1961) and shortly afterwards in plants (Frear and Swanson 1970) with the ability to detoxify atrazine in maize. In maize, GSTs equal up to 1% of the total soluble protein of the plant. GSTs have been intensively studied for their involvement in

herbicide detoxification and several GSTs are known responsible for herbicide tolerance in crops and resistance in weeds (see also Cole 1994, Table 2).

Plant GSTs share overall similarities with the corresponding enzymes in animals, and they have been found in both, cytosol and, quite recently also in membrane preparations (microsomes) of plants. There are several reports indicating that microsomal forms exist in spruce (Schröder and Belford 1996), onion (Schröder and Stampfl 1999) and in 60 other plant species (Pflugmacher et al. 2000). The cytosolic as well as the microsomal GSTs of all investigated species except one enzyme purified from maize (Dean et al. 1995) are dimeric enzymes consisting of subunits of the same or similar size between M_r 23 to 30 kDa. The determination of subunit sizes is in most cases deduced from SDS-PAGE separation and migration. However this size determination is critical as amino acid analysis has shown that most if not all GST subunits are actually identical in molecular size (212 to 240 amino acids). Most isolated GSTs are homodimers, but there is a number of distinct heterodimeric forms which are of considerable interest because they seem to be induced under conditions of hormone action and stress (see below).

For years discussions are going on about the classification of GSTs and GST subunits into groups. Whereas the system is quite established in animal physiology, presenting five distinct enzyme subunit classes *alpha*, *mu*, *pi*, *sigma* and *theta*, plant and bacterial GSTs have been proposed by many authors as to belong into the *theta* class. This *theta* class seems to be a primitive and ancestral gene family including GSTs from bacteria, insects, and plants. However, thorough investigations by Droog (1997) have shown that plant GSTs may well have similarities to *theta*, but are themselves again subdivided into at least two distinct gene families (Figure 2).

One family resembles the *theta* but may be different from the animal *theta* class, whereas other GSTs may be subsumed in a second family that has been called *tau*, in line with names being used so far. Indications for a third plant GST class *(zeta)* arise from the lack of homologies with a group of carnation GSTs that are only expressed during senescence (Marrs 1996). The gene structure of known plant GST has also been investigated. The three different structures found in plants have also led to attempts of a classification, which is overlapping with the above-described classification according to amino acid sequences. Several GST genes possess two introns at conserved positions. Most GSTs designated as *theta* according to sequence homologies belong to this group. A second group is made up of only carnation GSTs exhibiting nine introns. As mentioned above, carnation GST seem to be different from all of the so far examined genes and gene products. The third and largest group exhibits genes with only one intron at a conserved position, contains monocot and dicot GSTs, and represents members of the *tau* GST class. Summarizing, the one intron and the two-intron genes seem

to have co-evoluted even before plants have separated in phylogeny. Marrs (1996) bases her classification of GSTs functional aspects as well as on gene structure. She hypothesizes type I GSTs (with three exons and two introns) to be defence genes against oxidative stress and herbicides, type II GSTs (with nine introns) as ethylene and senescence related and the biggest group, type III GSTs (with two exons and on intron) to be responsive under heat shock, heavy metals and pathogen stress.

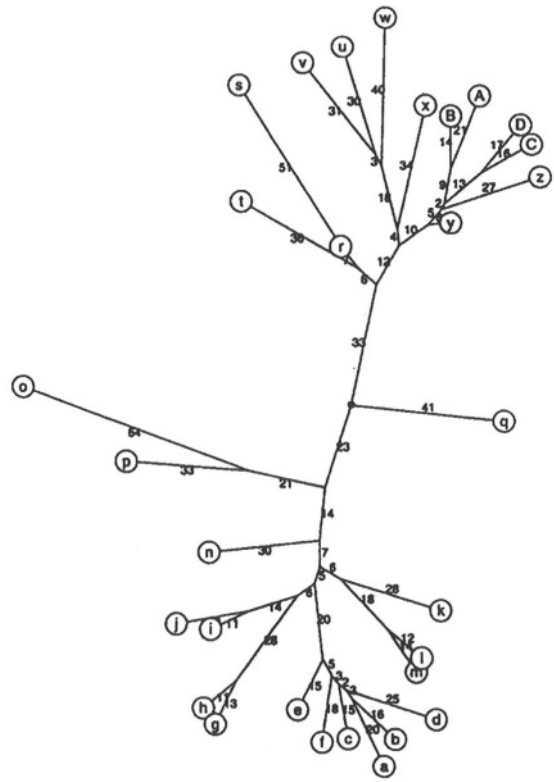

Figure 2. Classification of the so far available GST subunits according to sequence similarities (from Droog 1997). Sequences for this phylogenetic analysis of plant glutathione *S*-transferases were obtained from literature and from databases using the University of Wisconsin GCG software package (Devereux et al. 1984) and the Blast program (Altschul et al. 1990). Phylogenesis was done running the RootedTree option of the AllAll program at the ETH in Zürich. Abbreviations correspond to the following sequences:a: C7; b: Nt107, c: AtEST3, d: Egpar, e: GmGxI, f: Nt 114, g: MII-4, h: GmHsp26-A, i: PRP1-1, J: NT103, k: AtEST4, l: AT103-1a, m: At103-1b, n: CIP, o: Bz2, p: AtEST6, q: Dc gst, r:Bo gst, s: Pm239x14, t: ERD13, u: ZmGST29, v:ZmGST27, w: Ta gst A1, x: Zm GST26, y: AtEST1, z:Sc, A:parB, B:Hmgst-1, C:Atgst2, D:ERD11.

Genome projects have shown that individual plant species may possess more than 20 GST genes, but that the sequence homology between these genes is quite poor, in some cases even less than 10 % It is likely that more gene families will be identified in the future, as more GST sequences and genes become available.

Crystallization has been possible for few GST enzymes from animal and plant sources. In plants, so far only information is available on maize and *Arabidopsis* GST homodimers at 0.22 nm resolution. The pictures below (Figure 3) show impressively the enzymes architecture. Each of the two subunits is shown to consist of two distinct domains. The N-terminal domain is relatively smaller than the C-terminal domain. It exhibits a typical α/β-structure with a central four-stranded β-sheet flanked by two α-helices. The C-terminal domain is larger and consists of up to six amphipathic α-helices. This domain is linked to the N-terminal domain by linker segments that may vary in length between subunits. Structural differences are mainly located at the xenobiotic binding-site, the linker and within the C-terminus.

The subunits combine to form a globular protein with a more or less deep cleft in the contact area. This cleft contains the catalytic sites of the holoenzyme. Because subunits are more or less symmetrical in their molecular structure, the holoenzymes possess two catalytic sites, which have been reported to behave independently from each other (Mannervik and Danielson 1988). This may have considerable influence on the holoenzyme´s activity. Each of the catalytic sites of each subunit consists of two distinct regions. The first site is confined to the binding of glutathione and its analogues, called the G-site, and is highly specific for these tripeptides. The G-sites are orientated to the N-termini of each subunit. Experiments have shown that γ-glutamylcysteine will be accepted by this site but only at low rates, and that cysteine is not accepted at all.

Explanations for this typical behaviour have been deduced from the analysis of crystal structures of known GSTs, mostly from human or rat. All GSTs sequenced so far contain a glutamine or a glutamic acid residue that seems to be responsible for GSH γ-glutamic acid interaction (Reinemer et al. 1996). It seems that the orientation of the γ-glutamic acid residue inside the binding pocket of the protein is critical, and that the thiol group has to be placed quite specifically. The glycyl group is not such important for substrate recognition and can be substituted for or even lacking. According to the brilliant review of Wilce and Parker (1994) on this subject, the central aspect of the catalytic mechanism is the lowering of the pk_a of the glutathione thiol from 9.2 in aqueous solution to values between 6 and 7 when bound to the protein. As the resulting thiolate is much more reactive than the thiol group, a factor of 50 in reactivity towards nucleophiles may be gained by this activation (Kosower 1976). Sequence analysis of the G-sites indicates

several ways how the stabilization of the thiolate anion in the binding pocket may occur. In animal GSTs a highly conserved tyrosine residue may be crucial in this respect; in plants this role is taken over by a serine residue. The tyrosine or serine is obviously needed to act as a hydrogen bond donor promoting thiolate anion formation and putting the molecule into the ideal position for later substrate binding.

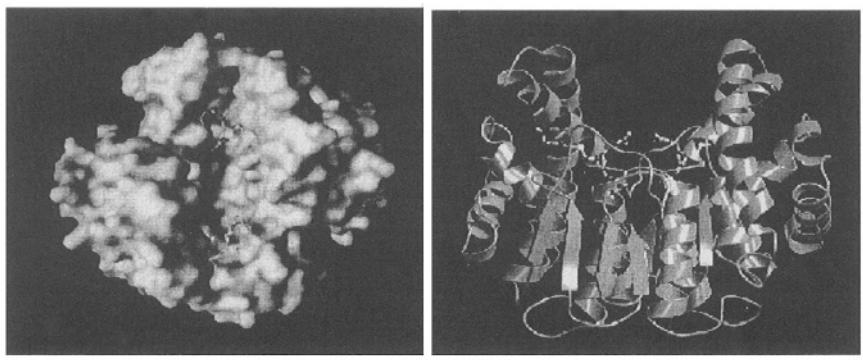

Figure 3. Left: Molecular surface image of *A. thaliana* GST; right: structure of *A. thaliana* GST at 0.22 nm resolution (from Reinemer et al. 1996, courtesy of Academic Press).

It has to be mentioned here that several authors have speculated in the past that the G-site has evolved from an ancestral gene coding for an universal glutathione binding protein. Wilce and Parker (1994) deduce that there are in fact similar topologies for glutathione binding in all glutathione dependent enzymes. However the expected sequence homology has not been found between classes. It may have been lost during evolution whereas the general architecture has been maintained.

The second ligand binding region responsible for the binding of the electrophilic xenobiotics, the H-site, is found more to the C-terminal region of the subunit as has been elucidated by photo-affinity labeling and deletion mutant studies in animal systems. Three-dimensional modeling has demonstrated that this site is orientated to the outward regions of the cleft and may form a flat pocket or a hollow, depending on subunit type. This has been confirmed by structure activity investigations for several animal GST binding sites. The H-site is hydrophobic but much less specific for substrate types allowing numerous substrates to bind. This is the reason why initial attempts to classify glutathione *S*-transferases according to substrate types (e.g. aryl- and alkyl-transferases and others, Habig et al. 1974) failed. When maize GSTs were tested for substrate specificities, at least 11 different forms

had been identified with multiple overlaps (Dean et al. 1991). CDNB, DCNB, pNBC, EPNP and ethacrynic acid were found to be partially overlapping substrates for conifer GST due to the different mechanisms of thioether formation (Habig and Jakoby 1981, Lamoureux and Rusness 1993, Schröder and Götzberger 1997). It is, however, possible that several isoforms of GST were not discovered since they did not exhibit activity for the compounds chosen (Izryk and Fuerst 1993). Novel data indicate that several GSTs are also active as glutathione peroxidases in *Alopecurus myosuroides* (Cummins et al. 1999) and in maize (Sommer and Böger 1999) utilizing a totally different molecular mechanism of activation for this catalytic reactions (Dean and Devarenne 1997).

Besides catalytic binding sites, GSTs have frequently been shown to possess noncatalytic binding sites. Especially in animal physiology, numerous hydrophobic compounds have been identified to bind to these pockets. Among them are bilirubin, heme, steroids, hormones and bile salts. This function has led to the name „ligandins" for several of the enzymes, and their function may be the intracellular inactivation or transport of the substances. Reports on non-catalytic binding in plant GSTs are few, but still significant metabolites have been identified as ligands: fatty acids, cytokinins and indole acetic acid (IAA). It is still speculative whether hormone binding occurs as a temporary detoxification reaction or as a special slow release storage form or whether it is a binding prior to intracellular transport. The responsible GSTs have been purified from *Nicotiana plumbaginifolia* leaves (Gonneau et al. 1998) and from the stem tissue *of Hyoscamus muticus* (Bilang et al. 1993), respectively. The auxin binding GST is inducible by several synthetic and natural auxins. Hence, the ligandin function would help unravel many phenomena connected to polarity of hormone transport in plants.

MECHANISMS OF GLUTATHIONE CONJUGATION

Conjugation reactions with glutathione have been reported for a vast number of compounds (see Table 1) and the catalytic mechanism has been clarified. For the GSTs, several mechanisms, including random, sequential and ping-pong have been proposed, but random binding order of substrates seems to prevail. However, from a physiological point of view glutathione binding should occur first because of the availability of glutathione in millimolar concentrations in the cells. This value is about three magnitudes larger than the dissociation constant between GSH and the enzymes (Wilce and Parker 1994).

Electrophilic centres necessary for GSH conjugation are found in arene-oxides, aliphatic and arylic halides, in α–β-unsaturated carbonyls, organonitro-esters and organic thiocyanates. Industrial substrates for GST are haloalkanes, chlorobenzenes, thiocarbamates, diphenylethers, triazines, chloracetanilide. In animals the oxidants acrolein, several propenals, lipid hydroperoxides, chlorambucil and fosfomycin are additional substrates.

For the xenobiotic binding there are in principle three different reaction types operating spontaneously or under catalysis by glutathione *S*-transferases (Figure 4, A-C).

Nucleophilic displacement of an alkyl or aryl halogen or a nitro-group seems to be the most frequently observed step. Conjugation of the herbicides atrazine, pentachloronitrobenzene (PCNB), or methidathion are examples for this type of reaction. Halogens or nitrogroups of these molecules are soft electrophiles and react readily with the GSH. In fact, the standard enzyme assays for glutathione *S*-transferase activity use 1-chloro-2,4-dinitrobenzene (CDNB) or 1,2-dinitro-4-chlorobenzene (DCNB) as substrates (Figure 5).

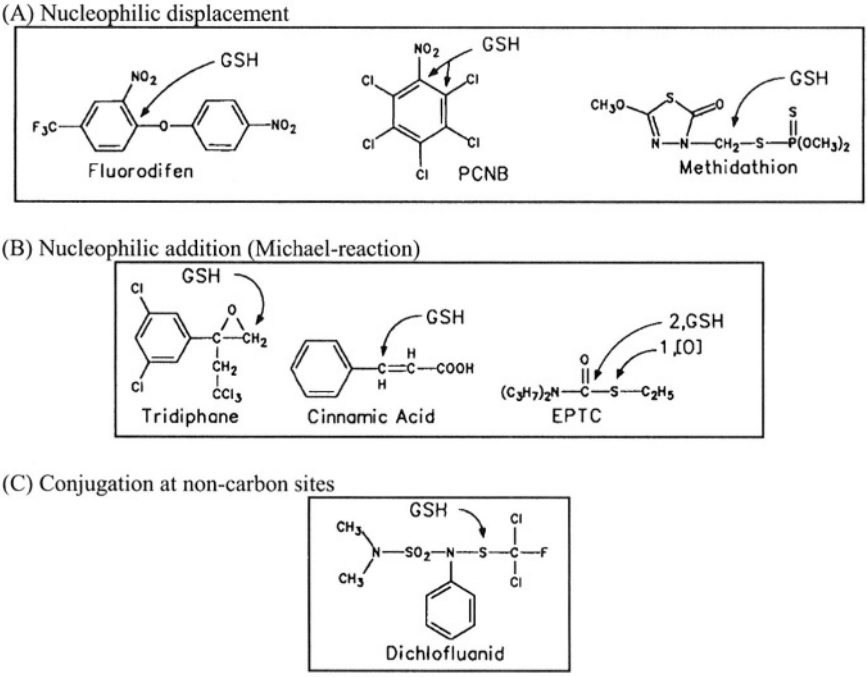

Figure 4. Mechanisms for the conjugation of xenobiotics with glutathione (from Lamoureux & Rusness 1993 modified).

Also to the substitution reactions belongs the detoxification of diphenylether herbicides (e.g. fluorodifen, fenoxaprop-ethyl). Here, an ether bond is cleaved and substituted by the thiolate. The phenyl residue is released and may be further conjugated with sugars.

Addition of the thiolate to carbon-carbon-double bonds has already been mentioned above. It is a special type of reactions on compounds with reactive carbon-carbon double bonds neighboured by an electron-withdrawing group (Talalay et al. 1988). Conjugation of tridiphane or cinnamic acid may be examples for this type of reaction (Lamoureux & Rusness 1986, Diesperger & Sandermann 1979). The conjugation on these bonds is a so-called Michael reaction and leads to a labile conjugate that may be sensitive to pH changes.

In plants, reactions with non-carbon sites have scarcely been described, e.g. as been reported for diclofluanid (Schupahn et al. 1981), but they may still be significant.

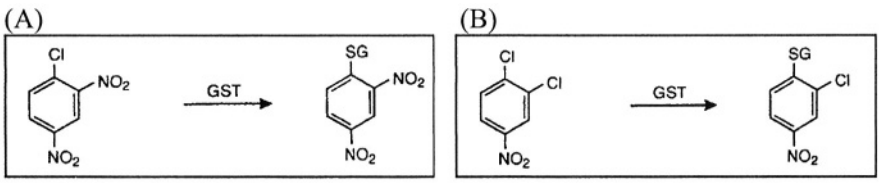

Figure 5. Conjugation of the standard substrates (A) CDNB, (1-chloro-2,4-dinitrobenzene) and (B) DCNB (1,2-dinitro-4-chlorobenzene) to glutathione. The assay conditions are easy to meet and measurement of conjugate absorption at 340 or 345 nm in a spectrophotometer provide fast success even for beginners in enzymology, however, these substrates do not cover all glutathione *S*-transferases. The recent identification of several novel isoforms of GST has to be ascribed to the fact that they were overlooked in previous studies only utilizing the standard substrates.

MODULATION OF GST ACTIVITY IN THE PLANT

GSTs are expressed in plants in a tissue specific manner, and reports are available on heterogeneous expression of isoenzymes in single leaves. The reason for this is probably found in an age specific expression of GST that has been pointed out in the context of inducibility of the enzymes under ethylene and during senescence. Maize leaves exhibit high GST activity toward atrazine in early development, but after the age of 14 days this activity is lost and not regained during leaf ageing (Hatton et al. 1996).

In this context, another question is raised by the observation that heterodimeric GSTs might appear under certain stress conditions. Heterodimeric GSTs have been described to possess mixed activities and specifici-

ties referring to the respective homodimers. Dimerization of subunits has been reported to occur spontaneously, but in artificial systems expressing the subunits heterologously, attempts failed to combine them randomly and to regain the expected activities (Dixon et al. 1999). The preliminary conclusion is that only subunit members of the distinct gene families will reconstitute to holoenzymes. However, as no database is available on the mechanism of induction or spontaneous rearrangement of GST subunits, this phenomenon might provide surprising future insights. The effect of subunit rearrangement on total GST activity might be massive.

Comparable to animal GST, plant GST activity may be enhanced by various xenobiotics, antidotes or natural compounds via the induction of distinct isoforms of the enzyme (Wiegand et al. 1986, Debus and Schröder 1990, Anderson and Gronwald 1991, Schröder et al. 1993).

An important property of GSTs is their inducibility by herbicide antidotes (safeners). This reaction has mostly been ascribed to monocotyledons and it is the decisive step conferring herbicide tolerance to many agricultural crops, but has also been observed in conifers. Wheat possesses more than eight GST isoforms when safener-treated. Five of them are heterodimeric and solely detected after the safener application.

Table 3. Sequence of steps leading to safener-induced herbicide tolerance (from Izryk and Fuerst 1997, modified).

Step 1:	Absorption of safener by seedlings into root and/or shoot
Step 2:	Metabolism of safener to active form by phase I enzymes
Step 3:	Specific signal recognition and signal transduction
Step 4:	Increased transcription of GSH synthesis and GST genes
Step 5:	Increased levels of GSH and GST isoforms
Step 6:	Increased herbicide conjugation
Result:	Enhanced whole-plant tolerance to herbicides

A hypothetical sequence of the events leading to an increase of herbicide tolerance in crops after safener application has been proposed by Izryk and Fuerst (1997), Table 3. From this it seems likely that several reactions take place in a more or less coordinate manner and may require a cascade process triggered by still unknown receptors somewhere in the cell. Halogenated air pollutants seem to act in a similar if not the same way on glutathione and GST in conifers. Unfortunately, the data basis is quite narrow, and only few reviews cover this subject (see Schröder 1998a,b).

Oxidative stress, but also anoxia and follow-up reactions like H_2O_2 evolution and the like are also known as inducers of plant GST (Table 4). Moreover, phytohormones and pathogens may act as inducers (Droog & al. 1993,

Mauch & Dudler 1993, Zhou & Goldsborough 1993). Natural and synthetic auxins seem to be very specific inducers of GSTs of the *tau* class in many plants, whereas *theta* gene products remain uninduced. Even more puzzling, it has been observed that in some cases gene activation is strictly specific for a single inducer, but in many cases evidence exists that seemingly unrelated and multiple stresses may as well lead to the activation of a single gene or a whole bundle of genes.

Experimental evidence points to a transcriptional regulation of the activation process, although the precise mechanisms have not been elucidated yet. Similar to safener-mediated induction, a reaction cascade has been postulated that is driven by a surplus of oxidative processes in the cytosol (Daniel 1993).

Table 4. Inducers of GST genes.

Inducers of GST genes			
Abscisic acid	dithiothreithol	giberellic acid	oxidative stress
Anaerobiosis	elicitors	GSH	ozone
Auxins	ethacrynic acid	H_2O_2	pathogens
Auxin analogs	ethanol	heavy metals	salicylic acid
Chlorocarbons	ethylene	herbicides	safeners
Cytokinins	flooding	jasmonic acid	substrates

This may be true for a number of GST, but is surely not valid for all isoforms. Some promoters of GST have been found to contain regulatory elements such as *ocs* (octopine synthase, Zhang and Singh 1994). These *ocs* elements seem to respond to electrophilic compounds in a manner analogous to the AP-1 elements of animals. Therefore, *ocs* elements may be involved in the observed changes in gene expression.

GST activities have been shown to be induced within few hours after contact with xenobiotics or hormones or the onset of stress. It seems that two mechanisms, activation and true induction are possible. The basis for rapid activation might be binding of activators at non-catalytic sites and/or the formation of heterodimers. Induction is thought to proceed via gene activation and *de novo* synthesis of protein (Schröder and Pflugmacher 1996).

In principle the inducibility of enzymes is nothing special, but it has to be considered that GSTs play a central role in the adaptive answers of an organism toward numerous stimuli. This even more as various inducers themselves are substrates of GSTs or become substrates after activation. Hence it sounds likely that GST occupy a key position for the induction of other related enzymes such as chinone reductases, glucosyl transferases and enzymes of glutathione synthesis as well as on the glutathione conjugate pump in the tonoplast.

In this context it has been speculated that GST and other phase II enzymes are induced by GST substrates (=electrophiles) but that the resulting GS-conjugates themselves interact with the homeostasis of glutathione pools (Hayes & Pulford 1995). On the contrary, studies with onion showed that GS conjugates have a pivotal role in the course of the intracellular signalling processes responsible for the activation of cellular defence, whereas γ-glutamylcysteine conjugates of the same compounds were somewhat inactive (Schröder and Stampfl 1999). These authors hypothesized that some glutathione conjugates serve as signal molecules being transported to the nucleus. Signal inactivation would be achieved by sequestering the conjugate in the vacuole.

Whereas most investigations on safeners and inducers focus on activation of GST, it is interesting to see that also inhibition and loss of GST activities may be encountered under the influence of xenobiotics. This has been shown repeatedly in conifers under the influence of air pollutants and other foreign compounds. Of course, such a loss of detoxification capacity for a series of electrophilic xenobiotics may have consequences on the fitness of plants in the environment and on their susceptibility for other stressors.

NATURAL FUNCTIONS OF GST

Natural functions for the GST have been discussed since their discovery. The ubiquitous distribution and the abundant presence of GST in various tissues of animals and plants may be a sign for their obvious importance. GST of mammals, especially rodents and human have been investigated thoroughly for their physico-chemical and catalytic properties (Mannervik und Danielson 1988, Picket and Lu 1989, Daniel 1993) because of their important role in toxin conjugation, drug metabolism and anticancer therapy. Natural functions of GST in plants have only scarcely been investigated (Table 5). However, there are several reports on the detoxification of toxic endogenous metabolites. For example, in wheat all eight characterized isoforms have activity with crotonaldehyde and an isothiocyanate. Furthermore, they exhibit all glutathione peroxidase activity as do certain maize GSTs. Phytohormones, fungal and bacterial toxins may also be conjugated, but experimental evidence is scarce.

The observation that GST genes belong to early response genes of auxin action and an association with cell division has led to a new view on the enzymes natural functions although catalytic functions have not been assigned. Conjugation of IAA or its analogues should however be excluded, as auxins (except for synthetic analogues with chlorine groups) do not carry

electrophilic centres. They are more suitable as substrates for the glucosyltransferases of plant cells.

Table 5. Possible natural functions of plant GSTs.

Postulated mechanism	Reference
Detoxification of lipid hydroperoxides	Mannervik et al. 1987, Marrs 1996, Sommer and Böger 1999
Conjugation of endogenous metabolites	Diesperger and Sandermann 1979, Edwards and Dixon 1991, Dean et al. 1995
Conjugation of phytohormones: IAA, ethylene, GA	Lamoureux and Frear 1987, Meyer et al 1991, Takahashi and Nagata 1992, Zettl & al. 1994
Detoxification of fungal toxins, pathogen defence	Dudler et al. 1991, Mauch and Dudler 1993
Donjugation of DNA-degradation products	Morgenstern et al. 1985
Regulation of GSH-pool	Lamoureux and Rusness 1989
Transport of (thio-)phenols, chlorophyllin, anthocyans	Martinoia et al. 1993, Singh and Shaw 1988, Marrs & al. 1995
Conjugation and transport of medicarpin and similar compounds	Li et al. 1997
Increase of drought tolerance	Dhindsa 1991
Antioxidative protective protein	Levine et al. 1994

REASON FOR SEQUESTRATION AND METABOLISM OF GS-CONJUGATES

Evidence for the inhibitory action of GS-conjugates on GST has been brought about by animal physiologists about a decade ago. Glutathione conjugates were recognized as competitive GST and GSSG reductase inhibitors in animals and plants (Ishikawa 1987, Ishikawa et al. 1994). In plants, evidence has been presented for the inhibition of corn and giant foxtail GST by the glutathione conjugate of tridiphane and other xenobiotic SG-conjugates (Lamoureux and Rusness 1989). The diuretic drug, ethacrynic acid (EA), is a reversible inhibitor of π-GST in animals (Ploemen et al. 1990, 1993, 1994) but also in plants (Schröder and Götzberger 1997). Norway spruce GSSG reductase (GR) has been shown to be inhibited at low concentrations of the model *S*-(p-nitrobenzyl)glutathione conjugate (Schröder and Wolf, unpublished). Lamoureux and Rusness (1989) report the inhibition of spinach GR by several glutathione conjugates including *S*-(tridiphane)glutathione, *S*-(2,4-dinitrophenyl)glutathione and *S*-hexyl-glutathione. Interestingly, *S*-(propachlor)-glutathione and *S*-(methyl)-glutathione were not inhibitory to this enzyme under identical experimental conditions.

Furthermore, reversibility of glutathione conjugation has been proven (Ishikawa 1987). Especially Michael-type additions to xenobiotics may become unstable when changes in pH occur, e.g. after transport into other compartments. Whether this is the principle underlying the transport of anthocyanins (Marrs et al. 1995) or medicarpin (Li et al. 1997) in the form of GS-conjugates into the vacuole via the GS-X-pump remains to be elucidated. GS-conjugates of anthocyanins have never been isolated and identified from plant cytosol or vacuoles. Besides chemical cleavage, the foreign compound may also be liberated by reverted action of GST itself under favorable conditions (Ishikawa et al. 1994).

The elimination of GS conjugates seems to be especially important in cases where the conjugation leads to bioactivation, as in the case of halogenated hydrocarbons which are known to be activated via GS-conjugation to genotoxic electrophiles reacting with guanidine in DNA (Anders 1988, Ishikawa et al. 1994, Dekant et al. 1986, 1987). Rapid translocation of GS-conjugates by multi-drug resistance (MDR) proteins in animals and other membrane ATPases is thought to prevent these deleterious effects (Ishikawa et al. 1994).

METABOLIC PATHWAYS OF GLUTATHIONE CONJUGATES

Although there are parallels in cleavage reactions of xenobiotic GS-conjugates and the catabolism of glutathione in animals and plants, there is considerable evidence that at least some of the enzymes of GSH metabolism are not identical with those for the breakdown of xenobiotic conjugates (Hubbell and Casida 1977, Frear et al. 1985, Anderson 1990, Lamoureux et al. 1991). In plants, the cleavage of glycine from the tripeptide has been addressed as the first step of GSH degradation (Figure 6). It is catalysed by a specific GSH-carboxypeptidase (Wolf et al. 1996). The remaining dipeptide, γ-glutamylcysteine, is further degraded to cysteine and glutamate via 5-oxoproline. This pathway does not depend on the activity of a γ-glutamyltranspeptidase as it does in animals, but on a γ-glutamylcyclotransferase. An 5-oxo-prolinase would, in the last step, catalyse the energy dependent formation of glutamate from oxoproline (Hubbell and Casida 1977).

Clear-cut evidence from numerous studies shows that glutathione conjugates are metabolized within hours to the corresponding dipeptide and subsequently cysteine conjugates (Lamoureux and Rusness 1980, 1983, 1989, 1993, Schröder et al. 1990). Contrary to animals, and in line with the breakdown of GSH itself, higher plants metabolize xenobiotic glutathione conju-

gates in a first step to γ-glutamylcysteine conjugates. The formation of γ-glutamylcysteine in animals (Bakke and Davidson 1994), as well as the production of cysteinylglycine conjugates in plants (see Table 1, Lamoureux and Rusness 1989, Riechers et al. 1996, Ezra and Stevenson 1986) seems to be a side reaction of minor importance. The resulting dipeptidyl conjugates are in both, animals and plants as well, cleaved to form cysteinyl conjugates. In animals this occurs via catalysis by a dipeptidase, but the nature of the respective enzyme in plants is obscure. The cysteine conjugates occupy a key position in the metabolic pathway. Only rarely have they been reported to be end points of the metabolism. In most cases they were in part or completely metabolized to an array of other products such as malonylcysteine conjugates, *S*-thiolactic acid derivatives, *S*-thioacetic acid conjugates and *S*-methyl-derivatives. A good example for this type of metabolism and the central role of the cysteine conjugate has been presented for the metabolism of fluorodifen in spruce cells (Schröder et al. 1990, Lamoureux et al. 1991, 1993, Figure 6).

Also *S*-malonyl-cysteine conjugates and their sulphoxides have long been thought to be final products of the metabolism of glutathione conjugates. They were hypothesized to occupy the same position in plant metabolism as mercapturic acids in animals. In analogy to the formation of malonylglucosyl conjugate formation it had been suggested that malonylation was the mechanism utilized to block further metabolism and a tag for tonoplast transport (Lamoureux and Rusness 1989). However, as malonylation is rather selective and not occurring on every cysteine conjugate (Lamoureux and Rusness 1983), it must be concluded that it is one of the more prominent phase III reactions in plants among others. In some cases, the liberation of the thio-group is prerequisite of subsequent glucosyl conjugation. Lamoureux and coworkers (Lamoureux and Rusness 1983, 1986, Schröder et al. 1990) were able to show that this cleavage reaction is catalysed by a C-S lyase which competes for the substrate with a malonyltransferase (see also Sandermann et al. 1997). Although numerous metabolism studies have been documented, the intracellular localization of the cleavage processes has not yet been elucidated.

COMPARTIMENTATION OF GLUTATHIONE CONJUGATES

Evidence for the sequestration of the glutathione conjugate of the chloroacetanilide herbicides, metolachlor and alachlor, has been presented with isolated vacuoles of barley (Martinoia et al. 1993, Li et al. 1995, 1997). The transport is energy dependent and distinct from other ATP-dependent mem-

membrane pumps in animals and plants. Wolf et al. (1996) were able to demonstrate that the GS-X pump operates *in vivo.* The affinity of the GS-X pump to its substrates is several times higher than the affinity of GST

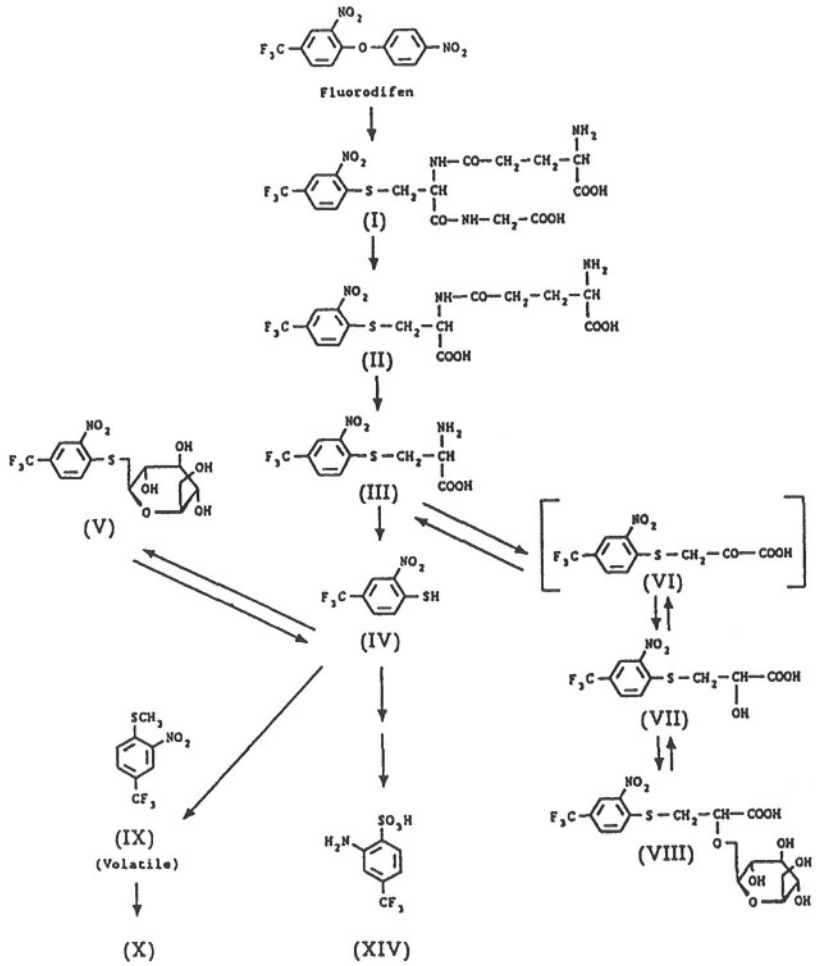

Figure 6. Metabolic pathway of glutathione conjugates formed from fluorodifen (from Schröder et al. 1990, Lamoureux et al. 1991, 1993, Schröder 1998a).

to the respective xenobiotic (Martinoia et al. 1993). Hence, it may be concluded that the rate-limiting step in xenobiotic removal is conjugation,

whereas the beneficial sequestration reactions proceed very fast and effectively.

The fact that many structurally diverse xenobiotics and natural compounds are transported via this GS-X pump into the vacuole leads to the point that glutathione conjugation may act as a „tag". It may be a general signal for the sequestration of structurally differing but functionally similar molecules from the cytosol. According to the above mentioned three phase model of xenobiotic metabolism, the GS-X pump in the tonoplast would be a constituent of the „excretion"-part of the metabolism in plants. Some authors have claimed the vacuole as final storage pool for all kinds of molecules (Marrs 1996, Martinoia et al. 1993, Li et al. 1995). As such, they characterize phase III as „storage excretion" in plants. However, this is not in accordance with the results of decades of research on xenobiotic metabolism in plants.

Moreover, work performed in onion indicates that GS-conjugates might be first transported to the nucleus before they get sequestered in the vacuole (see above).

On the background of these metabolism data, it is logical that glutathione conjugates may be intermediates rather than end products of detoxification. This topic has been reviewed extensively elsewhere (Schröder 1997). Elaborate investigations of herbicide metabolism in several plant cell cultures have elucidated that GS-conjugates have only short lifetimes in the cultured cells, and that they are rapidly further metabolized. First evidence for the enzymatic background of these cleaving reactions has been obtained only recently. Wolf et al. (1996) identified a specific carboxypeptidase for the cleavage of xenobiotic glutathione conjugates in the vacuoles of barley. Carboxypeptidases are exopeptidases cleaving terminal amino acids from polypeptides, whereas the physiological role of endopeptidases would be the regulation of enzyme activities via the cleavage of internal peptide bonds in polypeptides (Zuber and Matile 1968). A natural function for exopeptidases has been found in the cleavage of peptides in malt. Barley contains up to five distinct carboxypeptidases (Mikola 1983), three of which have been characterized recently (Breddam et al. 1983, 1985, Breddam and Sörensen 1987). However, none of these carboxypeptidases is identical to the enzyme purified by Wolf et al. (1996). After 1080-fold purification via cation exchange chromatography the enzyme was shown to be a 56 kD monomer. This enzyme has activity for the cleavage of glycine from several xenobiotic glutathione conjugates (Table 2) with good affinity. The carboxypeptidase does not accept conjugates of ethacrynic acid nor *S*-(alachlor)-γ-glutamylcysteine as a substrate, although the latter intermediate has been shown to be present in the vacuoles of [^{14}C]-alachlor treated barley leaves (Schröder and Wolf unpublished).

Only few glutathione conjugates have been shown to accumulate in the plant vacuole for more than few hours. The sequestration as such, however, makes a lot of sense because it efficiently lowers the bio-availability of the conjugate. Assuming vacuolar sequestration was a general mechanism for intermediate storage, a model for the compartimentation and enzymology of glutathione conjugates would look like the one depicted in Figure 7.

Carboxypeptidase seems not to be responsible for the cleavage of γ-glutamylcysteine residues. Consequently a dipeptidase or a γ-glutamyltranspeptidase should be present in the vacuole. These enzymes have been described for the catabolism of glutathione but have never been assayed with xenobiotic dipeptide-conjugates and their localization is not known.

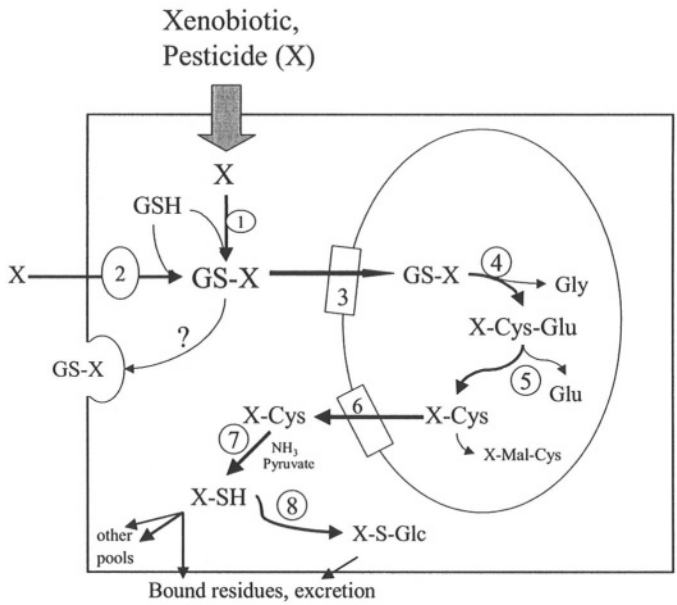

Figure 7. A model for the sequestration and enzymatic metabolism of electrophilic xenobiotics after initial glutathione conjugation. X: xenobiotic; GSH: reduced glutathione; GS-X: xenobiotic glutathione conjugate; X-Cys-Glu: γ–glutamylcysteine conjugate; Gly: glycine; X-Cys: cysteinyl-conjugate; X-Mal-Cys: malonylcysteine conjugate; X-SH: xenobiotic thiol; X-S-glc: *S*-glucosyl-conjugate. Involved enzymes: 1 cytosolic GST; 2 microsomal GST; 3 ATPase (conjugate carrier); 4 carboxypeptidase; 5 dipeptidase; 6 tonoplast carrier (hypothetical); 7 cysteine *S*-lyase; 8 *S*-glucosyltransferase.

Furthermore, and in order to explain the formation of secondary conjugates with sugars or even bound residue formation in cell walls, cysteinyl

conjugates should be re-transported into the cytosol. The GS-X pump is obviously not involved in this step (Schroder and Wolf unpublished). The export from the vacuole could be mediated as symport with protons or some other natural compound.

Cysteine lyase activity has been demonstrated in many organisms and is also present in plants (Mazelis and Creveling 1975, Lamoureux and Rusness 1980, Lamoureux et al. 1993). It seems to be a soluble, cytosolic enzyme. The resulting thiol metabolites may be secondary conjugated and tagged for the final fate of xenobiotic metabolites: export through the plasma membrane and storage in the cell wall, long range transport and excretion into the rhizosphere or volatilization and loss to the atmosphere.

CONCLUSIONS

Glutathione *S*-transferase and glutathione interact perfectly in protecting plants from electrophilic xenobiotics and endogenous toxic compounds. They are also involved into hormone metabolism and internal signal cascades. The enzyme class shows high flexibility and represents the starting point of a well developed metabolic pathway for the degradation and excretion of xenobiotic conjugates. Genomics and proteomics will provide more insight in the presence and activity of the GSTs in plants, and most important at all, about natural functions and their modulation under stress. Thus, glutathione and glutathione mediated metabolism of xenobiotics and agrochemicals play an important role in the adaptation of plants to the environment and to a man made pollution climate.

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Chapter 8

THE ROLE OF GLUTATHIONE IN PLANT REACTION AND ADAPTATION TO AIR POLLUTANTS

Luit J. De Kok and Michael Tausz

INTRODUCTION

The dramatic increase in the global human population and the concurrent need and consumption of fossil fuels for industrial production and transportation, the intensity of agricultural practice and animal husbandry have a significant impact on the chemical composition and quality of the atmosphere (Corell and Anderson 1991). It will consequence in a further increase in the levels of the so-called atmospheric greenhouse gases, CO_2, methane and various nitrogenous gases, which are suspected to result in a drastic global climatic change within the next 50-100 years (Bowes 1993). Despite the enforcement of legislature restrictions on pollutant emissions, in heavily populated and industrial areas the atmosphere is commonly polluted with a cocktail of inorganic and organic gaseous compounds and aerosols, which are a severe threat for natural and agricultural ecosystems. Definitely, air pollution is one of the major causes of the current decrease in biodiversity of plant species and economic losses in food production and quality (Fuhrer et al. 1997).

There is a wide variation in susceptibility of plant cultivars and species towards air pollutants. The physiological basis for the variation in air pollution response is still largely unresolved, despite the ample experimental information on the impact of air pollutants on plant metabolism. For some agents a distinction between acute and chronic plant injury can been made. Acute injury occurs upon exposure to a temporarily high peak level of the air pollutant and is characterized by the rapid occurrence of visible foliar injury, wilting or defoliation. The type and pattern of the development of visible foliar injury is often pollutant specific. Chronic injury occurs upon longer-term exposure to a lower level of the air pollutant and results in reduced biomass production, altered development and morphology, decreased fitness

D. Grill et al. (eds.), Significance of Glutathione to Plant Adaptation to the Environment, 185-205.

and/or reproductive output, higher susceptibility toward stresses and pests, whereas visible injury is absent or only visible after prolonged exposure. Some examples of the minimal effective concentrations of air pollutants for acute and chronic injury in plants are given in Table 1.

The nature and mode of the reaction of an air pollutant in the plant and the possible physiological response in order to prevent injury is strongly dependent on the chemical and physical characteristics of the pollutant. However, the interpretation of the available data is often complicated by a lack of information on the actual pollutant uptake rate (deposition rate) versus the plant response and by differences in the definition of air pollution injury. For the photochemical air pollutants O_3 and PAN (peroxyacetylnitrate), the physiological basis for injury is directly related to the high oxidizing potential of these gases. In this case, the difference between acute and chronic plant injury is often not that distinct. For sulphurous and nitrogenous air pollutants, the underlying physiological basis for acute and chronic injury is more complex and there are definitely differences in the mode of action and in plant response to either high peak level exposures on one hand, or prolonged low level exposures on the other hand. The impact of these air pollutants on plants is sometimes even paradoxical, since the deposited sulphur and nitrogen may be utilized as nutrient for plant growth.

Table 1. Minimal effective concentration range of some major air pollutant wherein plant injury may occur (derived from Posthumus 1998).

Air Pollutant	Chronic Injury ⇔ Acute Injury concentration [nl l^{-1}]
PAN	10
O_3	25
SO_2	10 ⇔30
H_2S	30 ⇔ 300
NH_3	15 ⇔ 350
NO_2	100 ⇔ 600

Glutathione and its homologues homoglutathione (in Fabaceae) and hydroxy-methylglutathione (in Poaceae) and the enzymes glutathione reductase (GR, B.C. 1.6.4.2) and glutathione *S*-transferase (GST, E.C. 2.5.1.18) function in various metabolic processes plant such as sulphur and selenium metabolism, modulation of enzyme activity and gene expression, and protection against oxidative and environmental stress (De Kok and Stulen 1993). In the present chapter the impact of air pollutants on glutathione metabolism will be evaluated. It will become evident that sulphurous pollutants may directly

interfere with glutathione metabolism, whereas glutathione, glutathione reductase, and glutathione *S*-transferase might have significance in the protection against toxic effects of photochemical air pollutants and differences in variation in susceptibility of plant cultivars and species to some of the air pollutants.

There is a large group of organic air pollutants, which also may affect plant functioning at low ambient levels, which will not be discussed in this chapter. The mode of action of these pollutants on plant metabolism is very diverse, but some, mainly the halogenated air pollutants may be metabolized/detoxified in plants in pathways similar to pesticides (xenobiotics). The conjugation of these xenobiotics with glutathione by glutathione *S*-transferases is of crucial significance for their detoxification (Schröder 1998; see Schröder in this volume).

The nitrogen pollutants NO and NO_2, as well as NH_3, will also be neglected in the present chapter. Although their toxicity on plants is well established, and their importance is evident, the relations to glutathione metabolism have been scarcely investigated yet. These pollutants are supposed to act either by a destabilization of nitrogen metabolism, acidification of the cellular compartments, and/or through free radical mechanisms (since NO and NO_2 are free radicals). Glutathione as a tripeptide is strongly connected to nitrogen and amino acid metabolism (Figure 1), and glutathione is an important component of the cellular defence system against free radicals. Hence, complex interactions of nitrogen pollution with glutathione may be expected and remain to be explored.

FOLIAR DEPOSITION OF GASEOUS AIR POLLUTANTS

The impact of air pollutants on plant function and the variation in plant susceptibility will largely depend on the actual pollutant doses, i.e. the rate of the pollutant uptake by (deposition to) the plant foliage. The foliar uptake of gaseous air pollutants is mainly determined by its diffusion through the stomates and its rate of absorption by the mesophyll cells (Mansfield and Freer-Smith 1984). The cuticle is hardly permeable for most air pollutants (Lendzian 1984) and the transfer through the cuticle is generally a negligible factor in the total deposition. The air pollutant deposition through the stomates can be described by Fick's law for diffusion:

$$J = \Delta c/g$$

Where J represents deposition of the air pollutant (pmol cm^{-2} s^{-1}); Δc the concentration gradient of the air pollutant (concentration difference between atmosphere and shoot interior; pmol cm^{-3}); g the diffusive resistance of the shoot towards the air pollutant (s cm^{-1}), which is predominantly determined by the stomatal and mesophyll resistance (Baldochi 1993, De Kok et al. 1998). The mesophyll (internal) resistance is dependent on the physical and biochemical factors of the pollutant, such as the solubility, the dissociation rate, the reactivity of the gas, and the rate of metabolism.

SULPHUROUS AIR POLLUTANTS

Occurrence and phytotoxicity

The atmosphere in rural areas contains only trace (ppt) levels of inorganic (SO_2, H_2S) and organic sulphur gases (carbon disulphide, carbonyl sulphide, dimethyl sulphide, mercaptanes). However, the concentrations are substantially higher in areas with volcanic activity and in the vicinity of industry or bioindustry. In case of SO_2 (and more locally H_2S), the ambient concentrations may exceed values at which effects on plant functioning are probable (Table 1). The physiological background of the phytotoxicity of sulphurous air pollutants is rather complex. The impact of SO_2 and H_2S on plant proves to be paradoxical, since these gases may both act as toxins and nutrients (De Kok 1990, De Kok and Stulen 1993, De Kok et al. 1998).

Chronic injury by SO_2 may be caused by (1) the negative consequences of acidification of tissue/cells upon after the dissociation of the absorbed SO_2 ($SO_2 + H_2O \rightarrow H^+ + HSO_3^- \rightarrow 2H^+ + SO_3^{2-}$) or, (2) by its reaction with cellular components that results in a disturbed metabolism, reduced growth and fitness. Visible injury at acute high levels of SO_2 may be the consequence of (1) severe cellular acidification or, (2) the direct effects of high endogenous toxic sulphite levels. Acute SO_2 injury also may be (3) the direct consequence of intracellular superoxide mediated free radical chain oxidation of sulphite to sulphate, which may occur in particular in the chloroplasts (De Kok 1990).

Chronic injury by H_2S may be caused by the disturbance of metabolism by the reaction of sulphide with cellular components. Metallo-enzymes appear to be particularly susceptible to sulphide, in a reaction similar to that of cyanide. The direct cause of visible injury and wilting which may occur at acute H_2S levels is still obscure. Similar to the observation with SO_2, also high endogenous sulphide concentrations may induce a free radical chain oxidation in chloroplasts (De Kok et al. 1983).

In contrast to the inorganic sulphur gases, there is still little information on the impact of most of the organic sulphur gases on plants and the impact of chronic low levels on plants functioning has barely been studied. In contrast to the observation with SO_2 and H_2S, acute high levels of carbon disulphide, carbonyl sulphide and methanethiol (1.8 – 3.6 µl l^{-1}) did not induce visible plant injury (Taylor and Selvidge 1984).

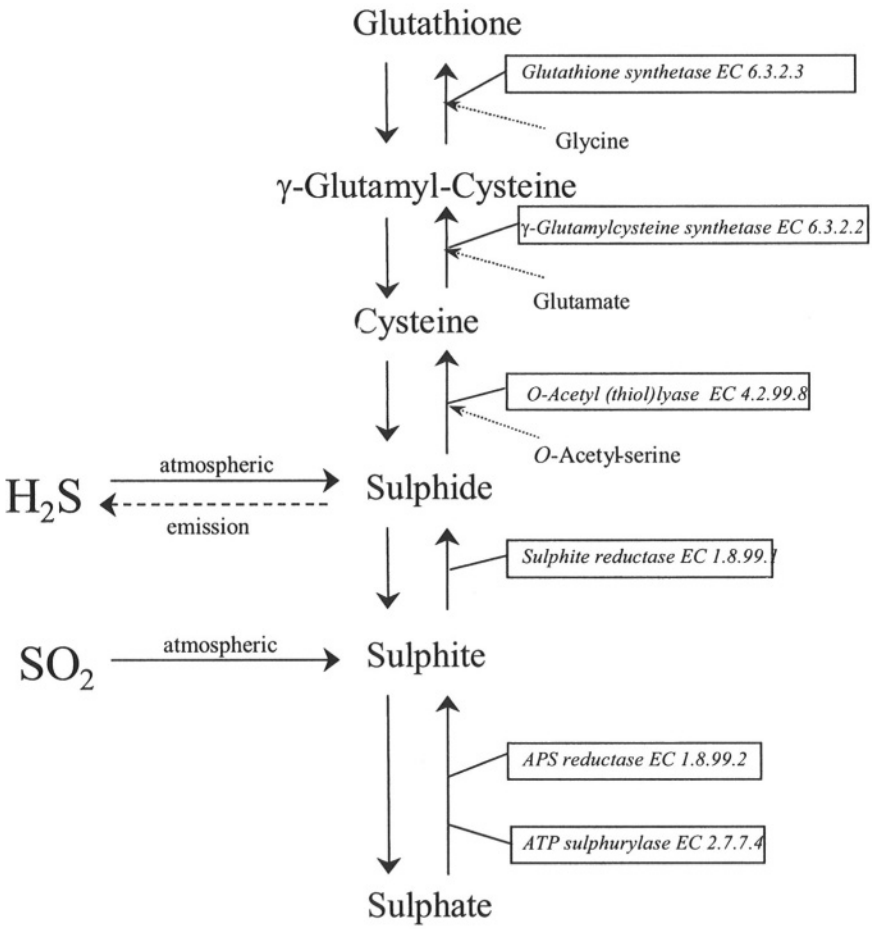

Figure 1. Simplified scheme of the relations of sulphurous air pollutants and the sulphur and glutathione metabolism in plants.

Impact on sulphur and glutathione metabolism

Sulphur is an essential element for plant growth. It has been recognized for several decades that foliarly absorbed sulphurous air pollutants may be utilized as sulphur source beneficial for plant growth. It has been estimated that dry sulphur deposition at atmospheric SO_2 (and H_2S) levels as low as 0.03 $\mu l\ l^{-1}$ may substantially contribute to the sulphur fertilization of plants (Stulen et al. 1998). From laboratory experiments it is evident that at sufficiently high atmospheric levels plants are able to grow with sulphur gases as exclusive sulphur source. Recently, it has become apparent that in Western Europe, and likely in several other regions, agriculture practice even has co-evolved with the dry and wet deposition of atmospheric sulphur originating from industrial emissions. The recent and ongoing reduction in industrial sulphur emissions have to be compensated by additional sulphur fertilization in order to avoid losses of crop yield and quality by sulphur deficiency (Schnug 1998).

Plant shoots form a sink for SO_2 and over a wide range there is a linear relation between atmospheric concentration and uptake rate. The deposition is generally directly dependent on the degree of stomatal aperture, since the internal resistance to SO_2 is low. SO_2 is highly soluble in the apoplastic water of the mesophyll, where it dissociates under formation of bisulphite (HSO_3^-) and sulphite (SO_3^{2-}). Sulphite may directly enter the sulphur assimilatory pathway and be reduced to sulphide, incorporated into cysteine, and subsequently into other sulphur compounds including glutathione (Figure 1 gives an overview of the possible impact SO_2 and H_2S on sulphate uptake and assimilation pathways). On the other hand, sulphite may be oxidized to sulphate, extra- and intracellularly by peroxidases, intracellularly by specific sulphite oxidases discovered recently in plants (Mendel & Hansch, unpublished results), or non-enzymatically catalysed by metal ions or superoxide radicals. The resulting sulphate can be assimilated thereafter. Furthermore, the excess of sulphate which is not directly metabolized is translocated into the vacuole, where it appears to be poorly accessible for remobilization and metabolism. The latter explains increased sulphate levels in shoots, which are characteristic for SO_2 exposed plants.

The pattern of H_2S uptake by plants differs distinctly from that of SO_2. The mesophyll (internal) resistance of the shoots to H_2S and its deposition appears to be directly dependent on the rate of H_2S metabolism into cysteine and subsequently into other sulphur compounds (De Kok et al. 1998, 2000). In contrast to SO_2, the uptake rates of H_2S show a saturation curve (Figure 2) which indicates that the rate limiting step for H_2S uptake is a metabolic process, probably the incorporation of sulphide into cysteine (Figure 1). There is strong evidence that *O*-acetyl-serine (thiol)lyase is directly responsible in the

sible in the active fixation of atmospheric H_2S by plants. Carbonyl sulphide (COS) may also be metabolized after hydrolysis to H_2S and CO_2, however, its atmospheric concentration is probably too low to have any significance as plant sulphur source (Kesselmeier and Merk 1993).

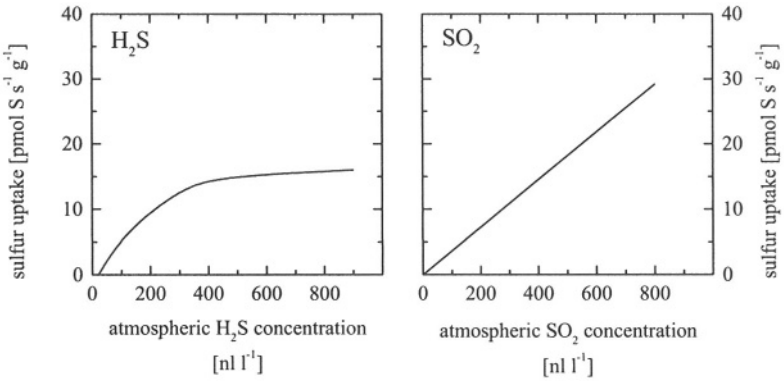

Figure 2. Uptake of atmospheric sulphur pollutants by young spruce trees as affected by atmospheric concentrations. Whereas SO_2 uptake is driven by outside concentration, H_2S uptake is limited by a metabolic process (scheme redrawn from original data in Tausz et al. 1998).

Part of the atmospheric sulphur, which is metabolized in excess of the normal requirement, may appear in the thiols (-SH groups) fraction in plant tissues (Grill et al. 1979, De Kok 1990, De Kok et al. 1998). In general, SO_2 and H_2S exposure result in enhanced thiol levels in shoots and in roots, which can already be observed within a few hours after the start of the exposure. In coincidence with the differences in metabolism between H_2S and SO_2 (Figure 1), the thiol accumulation is generally higher upon H_2S than upon SO_2 exposure at equal atmospheric concentrations. For H_2S it may be substantial at ambient concentrations as low as 0.03 $\mu l\ l^{-1}$. The thiol accumulation depends on the atmospheric SO_2 and H_2S level, but it may increase up to 5-fold and 2-fold in shoots and roots, respectively. The composition of the thiol pool may also be strongly modified upon SO_2 and H_2S exposure. The thiol pool in plants is dynamic and its level may strongly be affected by physiological and environmental factors, e.g. sulphur nutrition or stress impacts (Rennenberg and Lamoureux 1990, De Kok and Stulen 1993). The variation in the thiol concentration is mainly due to changes in glutathione content (or its homologues), the predominant thiol present in plant tissue

(typically > 90 % of total low molecular weight thiols). Upon exposure to SO_2 and H_2S also high levels of cysteine and, in darkness, of γ-glutamyl-cysteine may accumulate in the shoot, though the impact of SO_2 and H_2S on the composition of the thiol pool strongly varies between species. In roots, the thiol accumulation is generally exclusively due to enhanced glutathione levels (De Kok et al. 1998). After cessation of the exposure, the thiol level decreases very rapidly and normal values are reached again within one or two days.

The physiological background of the altered composition of the thiol pool in the shoot upon exposure to SO_2 and H_2S is not yet solved. Apparently, if the sulphur is directly supplied to the shoot and the regulation of sulphate uptake by the roots is by-passed, then there is no strict regulation of composition of the thiol pool in the shoot (De Kok 1990, De Kok et al. 1998). The absorbed atmospheric sulphur may even be metabolized outside of the chloroplast, wherein sulphur assimilation is located under normal conditions (Hell 1997). In the cytosol there might be a substrate shortage for the metabolism of the cysteine to glutathione. It has been observed that the accumulation of γ-glutamyl-cysteine in the dark can be prevented by adding the substrate glycine directly to the leaf tissue, which results in glutathione accumulation (Buwalda et al. 1990). A similar shortage of glycine for glutathione synthesis was observed in plants wherein the level of γ-glutamyl-cysteine synthetase was overexpressed (Noctor et al. 1997).

Thiol compounds such as glutathione are assumed to fulfil signal functions in the regulation of sulphur uptake and sulphur reduction in plants (Rennenberg and Lamoureux 1990, De Kok and Stulen 1993). Under normal conditions, sulphate uptake is in tune with the metabolic need for growth. The uptake may be regulated by changes in activity and/or the expression of sulphate transporter protein by negative feedback from sulphate itself or modulated by changes in concentrations of reduced sulphur compounds including glutathione (Davidian et al. 2000). SO_2 and H_2S impact studies are a promising tool to elucidate the signals involved in the regulatory aspects of the uptake, transport and reduction of sulphate in plants, and in the interactions between shoots and roots. Exposure of plants to sulphurous air pollutants may repress the uptake of sulphate by the roots (Figure 1; Brunold and Erismann 1974, Herschbach et al. 1995, De Kok et al. 1998) and its further transport to the shoots (Herschbach et al. 1995). It is still unclear to what extent an enhanced glutathione level in the roots is the trigger of the repression of sulphate uptake. Without regard of a normal sulphur supply to the roots, plants are able to switch in part to atmospheric H_2S taken up by the shoots as sulphur source for plant growth. However, the pattern of increase in glutathione levels in roots upon H_2S exposure (see above) appeared to be not in tune with the repression of sulphate uptake (Westerman et al. 2000).

Some plants may already accumulate sulphate in the shoot upon exposure to relative low levels of SO_2 or H_2S (De Kok 1990). It still needs to be resolved whether the accumulated sulphate originates from the oxidation of absorbed atmospheric sulphur gases in the plant foliage, or it reflects a poor shoot/root interaction in the regulation of the sulphate uptake by the roots and its transport to the shoot.

The plant foliage is the predominant site of sulphate reduction. It has been observed that SO_2 or H_2S exposure may result in a decrease of the activity of ATP-sulphurylase and adenosine 5'-phosphosulphate (APS) reductase (Brunold and Erismann 1974, Westerman et al. 2001), with sulphide, *O*-acetylserine or cysteine being the most likely regulators (Brunold 1990, Hawkesford and Wray 2000).

Glutathione and phytotoxicity of sulphurous air pollutants

Reactive oxygen species (ROS) can be formed at various sites in the plant cell, and even have important functions in plant metabolism, e. g. in lignin synthesis. The chloroplast is a major site of ROS formation, especially under conditions where the photosynthetic carbon fixation (Calvin cycle) is not in tune with photosynthetic electron transport (see Tausz in this volume). Under such conditions molecular oxygen may be photo-reduced at the site of photosystem I yielding superoxide (Asada 1999). Glutathione and glutathione reductase are of great significance in the protection of plants against the harmful effects of reactive oxygen species and free radicals. Glutathione may react directly with ROS, or it may stabilize and protect protein thiol groups by acting as a thiol buffer. Furthermore it plays a role as reductant in the enzymatic detoxification of H_2O_2 in the chloroplast in the ascorbate peroxidase-dehydroascorbate reductase-glutathione reductase cycle (De Kok and Stulen 1993, Kunert and Foyer 1993). Glutathione reductase catalyses the reduction of the formed oxidized glutathione, with NADPH as reductant (for more details see Tausz in this volume).

It has been postulated that an increase in glutathione concentrations and glutathione reductase (and superoxide dismutase) activities would have an adaptive value in the protection of plants against the toxic effects of SO_2 (Mehlhorn et al. 1986, Madamanchi and Alscher 1991, Soldatini et al. 1992). Part of the absorbed SO_2 would initiate a superoxide mediated free radical chain oxidation in the chloroplast, resulting in a burst of reactive oxygen species and other free radicals which could be the basis for the injurious effects of SO_2. Indeed, in vitro, in isolated chloroplasts the addition of relatively high levels of sulphite or sulphide resulted in a superoxide-triggered oxidation of these reduced sulphur compounds upon illumination (Asada and

Kiso 1973, De Kok et al. 1983, Ghisi et al. 1990, Dittrich et al. 1992). Light-induced oxidation of sulphite by chloroplasts was only substantial in broken chloroplasts (Ghisi et al. 1990) and was diminished by the addition of the electron acceptor of photosystem I (ferredoxin and NADP; Asada and Kiso 1973) or by the addition of superoxide dismutase or other scavengers of reactive oxygen species, glutathione included (Asada and Kiso 1973, De Kok et al. 1983, Ghishi et al. 1990, Dittrich et al. 1992). It is likely that sulphite/sulphide-induced superoxide mediated free radical chain reactions may have significance at acute high levels of sulphurous air pollutants, when the deposition substantially exceeds the plant potential to metabolize the absorbed sulphur and/or the oxidation potential of the extracellular peroxidases in the apoplast (Pfanz et al. 1990) and the antioxidative defence systems in the apo- and symplast. It may be one of the causes of the development of visible injury. Still it is unclear whether light is an essential factor for the development of plant injury by SO_2 (Olszyk and Tingey 1984). At normal ambient pollutant levels it is unlikely that high levels of sulfite occur in the chloroplast, since it would directly and with high affinity be metabolized as substrate in the assimilatory sulphate reduction (Figure 1). Even if significant amounts of sulphite entered the chloroplast, sulphite-induced radical formation in the light would be very unlikely. Intact chloroplasts are likely to contain sufficient active oxygen scavenging capacity to prevent light-triggered sulphite oxidation (Ghisi et al. 1990, Dittrich et al. 1992). Furthermore, the impact of SO_2 exposure on glutathione reductase levels are rather inconsistent (Tanaka et al. 1982, Grill et al. 1982, Madamanchi and Alscher 1991, Soldatini et al. 1992).

Chronically enhanced glutathione levels have even been suggested as one of the causes of the phytotoxicity of SO_2 (Grill et al. 1979). In this view it may result in a deregulation of cellular metabolism. The role of glutathione in its interaction with proteins is well known (Kunert and Foyer 1993). The glutathione redox system may also have an important role in environmental sensing and stress signalling within cells, within or between tissues, or even between organs (May et al. 1998). Enhanced glutathione levels beyond the normal metabolic control can have serious effects on the antioxidant system, which was demonstrated in transgenic plants with constitutively increased GSH concentration in chloroplasts. This did not stimulate antioxidant defence but, quite in contrast, promoted oxidative stress in chloroplasts (Creissen et al. 1999).

Another phenomenon that may involve glutathione is the development of chromosomal damages in root tissues of trees after the canopy has been exposed to sulphurous pollutants. This has been observed repeatedly upon both SO_2 and H_2S exposure (Müller et al. 1997, Wonisch et al. 1999a, b). The signalling pathway leading to this effect is enigmatic, but since glutathione

concentrations are enhanced in the needles, and glutathione is easily translocated in the phloem, it has been suggested as a likely candidate. Furthermore, the feeding of glutathione to fine roots of spruce trees indeed caused an increase in chromosomal damages in the meristematic tissues and abnormalities in the cell ultrastructure therein (Zellnig et al. 2000). A direct link between sulphurous air pollutants, enhanced tissue glutathione levels, and chromosomal damages in root meristems could not be established measuring tissue concentrations of GSH in the roots, because chromosomal damages were observed before GSH concentrations increased in the roots (Wonisch et al. 1999a). However, since average tissue concentrations may mask changes in certain cell types or even sub-cellular compartments, this result might not necessarily prove this hypothesis wrong. With the help of laser scanning microscopy and fluorescence labelling of glutathione it was shown that the meristematic region of root tips contain specifically high GSH concentrations which are important for the cell divisions therein (Sánchez-Fernanédz et al. 1997). The application of fluorescence microscopy in combination with digital image analysis also showed that the nuclei contain particularly high concentrations of glutathione (Müller et al. 1999, see image on the front cover). Changes of the glutathione system in the nucleus may be sufficient to cause the observed effects, and these changes can be easily overlooked when average tissue concentrations are investigated.

OXIDATIVE AIR POLLUTION – OZONE AND OTHERS

Occurrence and phytotoxicity

Oxidant air pollutants, among them the most abundant ozone, are formed in the atmosphere by the free radical driven interaction of precursors (hydrocarbons, nitrogen oxides, molecular oxygen) with solar radiation of wavelengths below 400 nm (Kley et al. 1999). Ozone is the best known of these substances and will be mainly discussed in the following. However, it must be kept in mind that oxidative air pollution is frequently occurring as a cocktail of many potentially toxic substances, for example peroxyacetylnitrate (PAN), aldehydes, peroxides, peroxy radicals and many others. This aggressive mixture of trace gases is commonly called photochemical smog.

The highest peak concentrations of ozone are found in urban regions with high irradiation and a high density of emittents producing the potential precursors (Kley et al. 1999), with the main culprit the burning of petrol products in traffic and industry. But also in remote areas ozone formation from long range transported precursors is facilitated in high elevations due to the

high radiation energy which leads to characteristic altitudinal gradients of atmospheric ozone concentrations in alpine regions (Smidt 1996).

Ozone is a strong oxidant highly reactive in aqueous and lipid phases. In water, a variety of reactive products, among them toxic oxygen species, may be formed by spontaneous reactions (Heath and Taylor 1997). In particular hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$), and the superoxide anion radical ($O_2^{\cdot-}$) have been linked to metabolic changes in plants (Sakaki 1998). Biochemically, ozone is able to oxidize a variety of cell compounds such as sulphydryl groups (also in glutathione) yielding disulphides, double bounds of fatty acids yielding carbonyl groups or hydroperoxides, amino acids and low molecular weight antioxidants, such as ascorbate and tocopherol (Hippeli and Elstner 1996, Mudd 1998).

The potency of ozone to induce acute or hidden injury in plants is well documented (Darrall 1989, Grulke 1999, Kley et al. 1999). Acute injury is mostly present as necrotic spots on the leaves whereas hidden injury leads to a decline in photosynthesis, carbon fixation and, consequently, growth. For some important agricultural plants and forest trees a relation between growth reduction and the ozone dose has been established from results of open-top chamber experiments (Fuhrer et al. 1997).

However, the primary site of ozone attack to leaf tissues and the mechanism of damage induction is still subject to discussion. Due to the reactive potency of ozone the site of the initial reactions would be expected close to the entry point. According to the principles given at the beginning of the present chapter the uptake of ozone to the leaves is governed by the flux through the stomata, since the cuticle is a near absolute barrier for this gas. Due to the reactivity of ozone with cellular chemicals the internal concentrations of this gas has been estimated as near zero by model calculations (Laisk et al. 1989). However, as ozone has no easily traceable isotope the actual dose of the pollutant, i. e. the amount which reacts with chemicals inside the leaf, has not been measured directly. It seems evident that ozone is able to reach the plasmalemma, but it is hardly capable of penetrating deep into the cell itself. The initial site of ozone action may thus be the apoplast, and the lipids and proteins of the membrane (Heath and Taylor 1997).

Although the phytotoxicity of ozone has been unequivocally demonstrated in many cases, the mode of action is still obscure (Mudd 1998). Several possibilities are discussed:

(1) Ozone easily degrades in aqueous phase forming reactive oxygen species (ROS), such as singlet oxygen, the hydroxyl free radical ($OH^{\bullet}$), superoxide anion free radical, or hydrogen peroxide (Sakaki 1998). Although plant cells are equipped with highly effective antioxidant defence systems (with prominent participation of glutathione, see Tausz in this volume) which scavenge these toxic molecules, at high ROS production rates the

defence capacity may be insufficient. As evidence for the involvement of ROS in ozone damage it has been reported that the pre-treatment of tissues with $O_2^{-\bullet}$ (superoxide) or $^1O_2$ (singlet oxygen) scavengers prevented ozone damage. The increase in $O_2^{-\bullet}$ in leaves treated with near-ambient levels of ozone was shown by EPR-signal analysis (Runeckles and Vaartnou 1997), and the action of singlet oxygen was demonstrated by chemiluminescence (Kanofsky and Sima 1995). Changes in the cell redox state may lead to several reactions, such as enzyme regulations or the activation of defence genes (May et al. 1998).

(2) Direct oxidative attack at the membranes may produce toxic secondary products, which can have a longer life span and may be transported to other locations, such as the chloroplasts. There they can inhibit enzyme activities of the Calvin cycle and lead to the measureable effects, such as the observed decreases in the photosynthetic rates (Heath and Taylor 1997). In combination with light energy this situation will lead to a production of ROS in the chloroplasts according to the scheme presented by Tausz in this volume (scheme in Figure 1 therein). The accumulation of long-lived toxic organic hydroperoxides was found in ozone treated plant tissues (Hewitt et al. 1990).

(3) An interesting interaction of the ozone induced free radical formation with a pathogen defence reaction, the “hyper-sensitive response”, possibly induces cell death (Sandermann 1996). The hypersensitive reaction of plant cells towards a pathogen includes the programmed death of the cells adjacent to the infection and thus inhibits the proliferation of the pathogen which needs living cells to feed on. Hydrogen peroxide and other ROS together with plant hormones are involved in the signalling processes leading to this plant response which is described in more detail by Foyer and Noctor in this volume. Ozone or the ozone generated ROS are thought to mimic the signal and 'erroneously' induce programmed cell death responses leading to ozone symptoms (Sandermann 1996).

Glutathione and ozone toxicity

Besides its role in plant sulphur metabolism glutathione is also an important component of the antioxidative system of the cell. Since the direct scavenging capacity of glutathione towards toxic oxygen species under physiological conditions is lower than that of ascorbate or tocopherol (Table 1 in Tausz in this volume), it is rather of importance as a protectant of protein -SH groups and it is essential to regenerate ascorbate from dehydroascorbate in the enzymatic glutathione-ascorbate cycle. The maintenance of the GSH/GSSG ratio is a prerequisite of the normal function of metabolism.

Many references report an increase in leaf glutathione concentrations upon experimental fumigation with ozone in various plants, such as conifers, deciduous trees, and also herbaceous plants (Alscher 1989). A detailed literature review of ozone effects on the antioxidative system of plants is given by Polle (1998). Mostly, changes in GSH concentrations were accompanied by increases in concentrations of other components of the antioxidative system, such as ascorbate or α-tocopherols. Ozone fumigation is also able to induce the enzymes of the glutathione-ascorbate cycle (Polle 1998, Sehmer et al. 1998) which supports a primary role of the antioxidant activity in response to ozone. Changes in the redox state of the GSH/GSSG pools can also be induced by ozone, in particular in sensitive cultivars, and under high ozone concentrations (more than 100 $\mu g\ m^{-3}$). In tobacco, the well-known and extensively studied ozone-sensitive cultivar BelW3 showed a more pronounced oxidation of the GSH redox pool than more resistant varieties and the GR activity declined. Spinach leaves showed a nearly complete oxidation of the GSH pool upon acute ozone stress (Luwe et al. 1993). However, the reports of ozone effects on the glutathione systems are very divergent (Polle 1998) and depend on many factors. As an example, spruce trees showed no response of the antioxidative systems to a long-term fumigation with about double-ambient ozone concentrations without regard of measurable effects on cell divisions (Wonisch et al. 1998, 1999c).

Increased contents of glutathione were also observed in trees subjected to high elevation stress in the field, a stress complex that is composed of high irradiation, low temperatures, and of high atmospheric ozone concentrations. A clear altitudinal gradient of glutathione contents in conifer needles was found in several studies in the Alps (Rennenberg et al. 1997, Tausz et al. 1997). However, in most field studies it is impossible to distinguish between different environmental factors, all of which are potentially inducing oxidative stress. Recent field study results on *Pinus ponderosa* growing along a pollution gradient in the San Bernardino Mountains (Southern California) partly clarified this aspect. In this region, trees at higher elevations are exposed to lower ozone doses, but to a higher natural stress level, whereas ozone load is greatest at the lower elevated plots. The trees under higher natural stress impacts contained more glutathione, i.e. a confirmation of the results found on spruce at elevation gradients in the Alps. On the other hand, the reduction state of the glutathione pool was significantly lower at the high ozone plots (Tausz et al. 1999a), indicating the crucial role of glutathione regeneration under ozone stress (Tausz et al. 1999b, also postulated by Luwe 1996, see below).

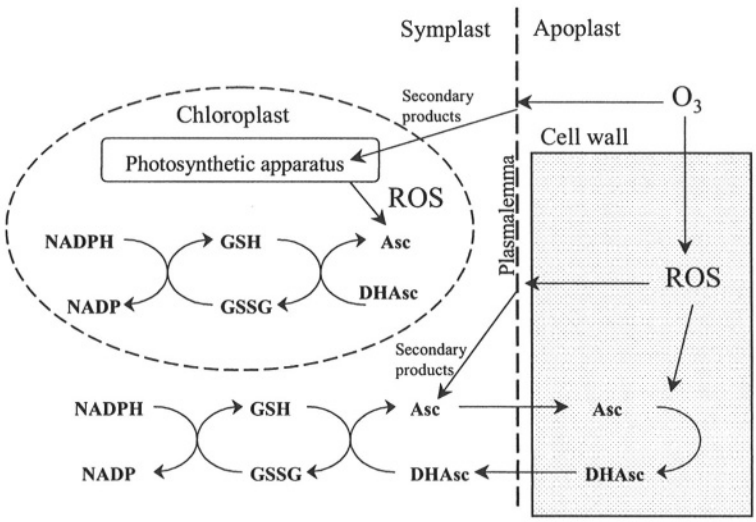

Figure 3. Compartmentation of ozone-induced ROS formation in plant cells. The apoplast contains considerable amounts of ascorbate but not glutathione. Glutathione takes part in ascorbate regeneration in the cytoplasm or in the chloroplast. GSH glutathione, GSSG oxidized glutathione, Asc ascorbate, DHAsc dehydroascorbate, ROS reactive oxygen species.

Recent experiments were conducted with transgenic plants containing manipulated activities of the glutathione homeostasis metabolism (Lea et al. 1998, Foyer et al. 1998). Poplar trees containing increased concentrations of foliar glutathione or increased activities of glutathione reductase were investigated (Noctor et al. 1997). Exposure of these plants to acute ozone levels demonstrated that constitutively increased pool sizes of glutathione did not ameliorate ozone resistance. Ozone resistance did not correlate with glutathione content or redox state in this system. Although elevated GR activity in the chloroplasts prevented the ascorbate pool from being oxidized, this did not lead to increased ozone resistance (Noctor et al. 1997).

As described above, on its way towards the plasma membranes ozone has to pass through the cell wall. The cell wall contains antioxidative compounds able to scavenge reactive oxygen species (Polle 1998). Peroxidases, which are involved in lignin production, represent the main antioxidative capacity in the apoplastic space, but also ascorbate plays a considerable role. The presence of glutathione in the apoplast is negligible. In spruce needles between 0 and 0.1 nmol total apoplastic GSH g^{-1} needle dry weight were reported by Kronfuß et al. (1998): The same needles contained between 0.1

and 0.7 μmol ascorbate g^{-1} needle dry weight in the apoplastic space. In other studies, GSH was unmeasurably low in the apoplastic space, such as in beech trees (Luwe 1996) or herbaceous plants (Lyons et al. 1999, in *Plantago major*). These findings confirm that in contrast to ascorbate, glutathione is not an important factor in the 'first line of defence', the antioxidant system of the cell walls (Polle 1998). However, since ascorbate is mainly regenerated through the action of the enzymatic ascorbate-glutathione cycle (Figure 3), cytoplastic GSH is indirectly involved in the apoplastic detoxification capacity of ascorbate. Since the presence of the enzymes of the ascorbate-glutathione-cycle in the apoplast is questionable, the dehydroascorbate formed by oxidation of ascorbate must be transported into the cytoplasm and regenerated therein (Figure 3). A corresponding ascorbate/dehydroascorbate translocation system in the plasmalemma exists (Horemans et al. 1998). This process involves the cytoplasmatic glutathione pool and might require adaptive responses thereof. There are some hints that the regeneration of apoplastic ascorbate is a time-consuming process and that therefore an oxidation of the tissue GSH pool may occur with a certain lag phase after ozone episodes (Luwe 1996) or the redox state of the glutathione pool may change upon long-term exposure (Tausz et al. 1999b).

The explanation of such divergent results may be found in the multiple roles glutathione may play in the response to ozone stress. Even in its role as antioxidant directly involved in the scavenging of ROS it is only one part of a complex system, and the specific conditions may require adjustments in the glutathione system (which are measurable as plant responses) or rather in other parts of the defence systems. The glutathione redox pool can play a role in environmental sensing and signalling of stress situations (May et al. 1998). In particular, transient changes of the GSH/GSSG redox state may be the signal for the induction of several defence reactions (Foyer and Noctor, Tausz in this volume). Glutathione is also involved in the pathogen defence reactions (Gullner and Kömives, Foyer and Noctor in this volume) that have much in common with ozone responses of plant cells (Sandermann 1996).

Furthermore, an appealing possibility is the involvement of the GSH redox system in long distance signal transduction of pollution stress impacts. Just like sulphurous pollutants, ozone applied to the canopies of spruce trees produces chromosome damages in the root meristems (Müller et al. 2000), often before biochemical or physiological effects in the canopies could be observed (Wonisch et al. 1999c). The signal transduction pathway in these cases is equally enigmatic as upon sulphurous pollutants, but glutathione as an easily transportable compound may be a probable candidate (Zellnig et al. 2000). As for sulphurous gases, for ozone effects a temporal correlation of chromosome responses and total fine root concentrations of glutathione could not be established yet (Wonisch et al. 1999a).

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Chapter 9

THE ROLE OF GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES IN PLANT-PATHOGEN INTERACTIONS

Gabor Gullner and Tamas Kömives

INTRODUCTION

The most abundant non-protein thiol compound in plants is the tripeptide glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) (Rennenberg 1997). Additionally structural homologues of GSH are found in plant tissues. In bean, soybean and some other leguminous plants (*Leguminosae*), homoglutathione (hGSH, γ-L-gutamyl-L-cysteinyl-β-L-alanine) was detected in considerable amounts (Klapheck 1988). Beside GSH, substantial amounts of hydroxymethyl-glutathione (γ-L-gutamyl-L-cysteinyl-L-serine) were detected in several species of the *Gramineae* family, for example in wheat, barley and rice (Klapheck et al. 1992, Zopes et al. 1993). Some other thiol peptides are also known (Meuwly et al. 1995). In most plant tissues, GSH is predominantly present in its reduced form, but the oxidized, disulphide form of glutathione (GSSG) can also be detected. The regeneration of GSH from GSSG is catalysed by the glutathione reductase enzyme (GR, E.C. 1.6.4.2.), which maintains the high GSH/GSSG ratio in plant cells (De Kok and Stulen 1993).

GSH and its homologues exhibit high chemical reactivity due to the sulphydryl group of their cysteine moiety. They are involved in various metabolic processes and are essential components of antioxidative and detoxification systems in plant cells. GSH can react both as a reducing agent and as a strong nucleophile, participating in the elimination of reactive oxygen species (ROS) via thiol-disulphide redox reactions, and in detoxification of various xenobiotics by conjugation reactions, respectively (De Kok and Stulen 1993, Mauch and Dudler 1993, Kömives et al. 1998, Noctor et al. 1998). The accumulation of GSH has been observed in various plants exposed to a wide range of abiotic stress effects. Several lines of evidence suggest that GSH and its homologues play major regulatory roles in biochemical-

D. Grill et al. (eds.), Significance of Glutathione to Plant Adaptation to the Environment, 207-239.

physiological responses of plants to various abiotic stresses including adverse environments and exposure to herbicides or heavy metals (Kömives et al. 1998, May et al. 1998a, Cobbett 2000, Foyer and Rennenberg 2000, Tausz and Grill 2000).

Plants are continuously exposed to attack by microbial pathogens. Successful pathogen invasion and disease (compatibility) ensue if the preformed and inducible plant defence mechanisms are inappropriate. In many cases the microbial invasion fails (incompatible interactions). Incompatibility can be due to preformed plant structural barriers or toxic compounds, or to the mobilization of effective defence mechanisms, which are able to prevent the establishment and spread of the pathogen. Bacterial, fungal and viral pathogens elicit a vast array of biochemical defence responses in plants after the recognition of the attacking pathogen. These responses are active processes involving a series of host gene expressions. A major difference between resistance and susceptibility to pathogens is in timing and magnitude of induction of host-defence genes (Király et al. 1991, Levine et al. 1994, Hammond-Kosack and Jones 1996, Zhu et al. 1996, Fritig et al. 1998). In recent years it has been found that GSH plays important regulatory roles in almost all of these defence responses (May et al. 1996a, Fodor et al. 1997, May et al. 1998a, Foyer and Rennenberg 2000). This review is an attempt to recapitulate our current knowledge about the role of GSH in plant defence reactions against microbial pathogens.

CHANGES IN CELLULAR GSH CONTENT AFTER INFECTIONS

The accumulation of GSH in plants exposed to various abiotic stresses has been reported in a number of papers (for reviews see: Noctor et al. 1998, Foyer and Rennenberg 2000). However, relatively little information is available about alterations of endogenous GSH levels in plants infected with microbial pathogens. The first report on this subject demonstrated the increase of GSH and hGSH levels in alfalfa and bean cells as a consequence of fungal elicitor treatment (Edwards et al. 1991). Some years later the changes of endogenous GSH levels were investigated in oat leaves infected by four *Drechslera* species having differing virulence levels. The necrotrophic fungal pathogen *Drechslera* (*Helminthosporium*) is the causal agent of oat leaf blotch. Marked reductions in GSH content starting approximately 2 days post-inoculation was observed in leaves infected by two highly virulent fungal species, while no changes were observed with the weakly virulent pathogens. It was concluded that decreases of GSH levels showed a progressively diminishing capacity of the leaf tissue to resist oxidative stress and to repair

damage by regenerating protein sulphydryl groups. It was also suggested that ROS are not part of the plant defence, but contrarily, they may facilitate the colonization of oat leaves by the virulent fungi (Gönner and Schlösser 1993).

Changes in GSH content were investigated also in the leaves of three near-isogenic tomato plants carrying either the resistance genes *Cf-2* or *Cf-9* or no *Cf* genes against the fungal pathogen *Cladosporium fulvum.* Leaves were infiltrated with a *C. fulvum* elicitor, which contained functional products of the fungal *Avr9* and *Avr2* avirulence genes. The flooding of the apoplastic domain of tomato leaves with the injected elicitor gave a synchronous activation of resistance responses. Total glutathione levels began to increase 2 and 4 hours after the injection of elicitor into the incompatible *Cf-2* and *Cf-9* leaves, respectively, and by 48 hours reached 665 % and 570 % of initial levels. A large proportion of this accumulation was the oxidized form, GSSG. The kinetics of GSH and GSSG accumulation followed closely the kinetics of changes in the levels of superoxide and lipid peroxidation. When the ambient relative humidity was increased to 98 %, increases in GSH levels were strongly delayed and attenuated. In the compatible interaction (in leaves containing no *Cf* gene) only a slight increase of total glutathione levels was observed. Marked increases in the level of GSSG are strong evidence that the elicitor treatment results in severe oxidative stress in a *Cf- Avr* gene-dependent manner (May et al. 1996a).

The fungal *Avr9* gene encodes an extracellular cysteine-rich, 28-residue protein (AVR9). This protein, which is a race-specific elicitor, contains three disulphide bridges and displays structural homology to cystine-knotted peptides, such as proteinase inhibitors. All disulphide bridges are indispensable for the necrosis-inducing activity. The correct folding of the protein was sensitive to the thiol-disulphide redox potential (GSH/GSSG ratio) *in vitro* (Van den Hooven et al. 1999).

The generally supposed protective role of cellular GSH was challenged by results of May et al. 1996b obtained with an *Arabidopsis thaliana* mutant. The *A. thaliana* mutant *cad2-1*, whose total glutathione content was only 30 % of the wild-type level, still exhibited an almost normal resistance level against an avirulent strain of the fungus *Peronospora parasitica.* This incompatible interaction resulted in a maintained stimulation of GSH synthesis in the mutant leaves up to a 2.5-fold increase of the GSH level. Concurrently, significantly increased GSSG levels were also observed indicating a perturbation in the cellular redox state during the incompatible interaction. Infection of the wild type resulted in a weaker (1.3-fold) and transient increase of GSH levels. The development of a virulent strain of *P. parasitica* was identical in both the mutant and wild type plants, without GSH accumulation. The responses of the *cad2-1* mutant and the wild type were similar also after infections with virulent and avirulent strains of the bacterium

Pseudomonas syringae pv. *tomato.* The results showed that severe depletion of GSH did not impair the capacity of *A. thaliana* to resist an avirulent pathogen and did not lead to greater susceptibility to a normally virulent pathogen (May et al. 1996b). The marked inducibility of GSH synthesis in the mutant during the incompatible interaction suggests that the rapid accumulation of GSH can compensate for its low steady-state level and sufficient GSH is present to quench induced oxidative stress.

Non-protein thiol levels were investigated in three barley cultivars following inoculations with the obligate biotrophic fungus *Blumeria graminis* f. sp. *hordei* (formerly called as *Erysiphe graminis* f. sp. *hordei*) that causes powdery mildew. Barley leaves contain significant amounts of hydroxymethylglutathione in addition to GSH (Klapheck et al. 1992), therefore the foliar non-protein thiol level was measured instead of GSH in mildewed barley leaves. Thiol levels moderately increased in leaves of a resistant cultivar and a moderately susceptible cultivar following fungal inoculation. However, no alteration of foliar thiol levels was found in mildewed leaves of a highly susceptible cultivar (El-Zahaby et al. 1995). Some years later the role of antioxidants in barley - powdery mildew interactions was investigated in a more detailed manner. Leaves of two barley isolines, a resistant line showing hypersensitive race-specific resistance to avirulent races of *B. graminis* and a susceptible line, were inoculated with *B. graminis* f. sp. *hordei.* Total leaf and apoplastic antioxidants were measured 24 hours after inoculation when maximum numbers of attacked cells showed hypersensitive death in resistant plants. GSH was present only in small amounts in the apoplast (1-2 % of the total GSH pool). GSH content of susceptible leaves and apoplast decreased, whereas that of resistant leaves and apoplast markedly increased after fungal inoculation, but the redox state was unchanged in both cases. These results showed that differential antioxidant deployment between compatible and incompatible interactions may be central to resistance strategies and that GSH might act as a signal-transducing molecule involved in the elicitation of defence responses (Vanacker et al. 1998a). Similar studies were carried out in three oat (*Avena sativa* L.) lines, which expressed different degrees of race non-specific resistance to infection with *B. graminis* f. sp. *avenae.* Inoculation caused a substantial increase in foliar GSH levels in two resistant lines but not in a susceptible one 24 hours after inoculation. No marked accumulation of GSSG was observed. The apoplast of uninfected oat lines contained measurable amounts of GSSG but GSH was not detectable. Following fungal inoculations, however, marked amounts of apoplastic GSH were measured in a resistant cultivar and the GSH/GSSG ratio substantially increased. GSH turnover and partitioning were modified during expression of non race-specific resistance (Vanacker et al. 1998b). Substantial amounts of antioxidative enzymes, including GR were also found in the apoplasts of both bar-

ley and oat leaves. The apoplast, the extraprotoplastic matrix of plant cells including the cell wall, provides a liquid interface between the environment and the plasma membrane of plant cells. The constituents of the apoplastic fluid play decisive roles in signal transduction leading to resistance (Vanacker et al. 1998c).

Inoculation of tomato leaves with the necrotrophic fungal pathogen *Botrytis cinerea* resulted in a significant progressive decrease of GSH levels while the GSH/GSSG ratio remained unchanged. The first visible symptoms, brown localized lesions, appeared 3 days after inoculation on the leaves. Two days later the fungus started to sporulate, a subset of lesions developed into spreading lesions and a typical grey mould appeared. ROS produced during the interaction are instrumental for *B. cinerea* to kill the host tissue in initial stages of infection. The accumulation of ROS may lead to strongly decreased foliar GSH levels, which seems to be a limiting factor for operation of the ascorbate-GSH cycle during the advanced stage of infection development, before the appearance of spreading lesions (Kuzniak and Sklodowska 1999).

Viral infections also can lead to marked elevation of foliar GSH levels. Tobacco mosaic virus (TMV) infection brought about a substantial elevation of GSH levels in both the infected lower and non-infected upper leaves of resistant tobacco (*Nicotiana tabacum* L *cv.* Xanthi-nc) plants (Fodor et al. 1997). Interestingly, the GSSG content (and the GSSG / total glutathione redox ratio) decreased in the infected leaves, which was probably due to the strongly induced GR activity in infected tissues. In the uninfected upper leaves the elevation of GSH levels occurred two weeks after TMV infection of the lower leaves and this elevation was concomitant with the development of systemic acquired resistance (SAR). The injection of salicylic acid (0.8 mM) into tobacco leaves also resulted in elevated foliar GSH content. It is known that the accumulation of salicylic acid is essential for the development of SAR (Ryals et al. 1996). One can suppose that the systemic induction of GSH-related antioxidative systems, together with other antioxidants, contribute to the appearance of SAR by the increased capacity of scavenging ROS during a second (challenge) infection (Fodor et al. 1997).

Summarizing the above reports it seems that the accumulation of GSH occurs mainly in incompatible plant-pathogen interactions. Incompatible interactions usually lead to a marked oxidative burst in plant tissues (Lamb and Dixon 1997). It is conceivable that the higher demand for antioxidative defence reactions leads to increased GSH biosynthesis in plant cells. Further studies, primarily the comparison of compatible and incompatible interactions are necessary to gain a deeper insight into the supposed protective role of GSH. The signalling mechanisms involved in induction of GSH biosynthesis during pathogen attack are unknown (Foyer and Rennenberg 2000). GSH is synthesized in a two-step reaction sequence (Rennenberg 1997, May et al.

1998a). In the first step, a dipeptide is synthesized from L-glutamate and cysteine by the enzyme γ-glutamylcysteine synthetase (γ-ECS, E.C. 6.3.2.2.). In the second step glycine is added to the C-terminal end of the dipeptide, in a reaction catalysed by glutathione synthetase (E.C. 6.3.2.3.). Jasmonic acid, which is a stress-inducible endogenous regulator (Wasternack and Parthier 1997), induced the accumulation of mRNAs encoding the enzymes of GSH biosynthesis without increasing GSH content in *Arabidopsis* (Xiang and Oliver 1998). Several plant species, such as *Arabidopsis,* Indian mustard, poplar and tobacco have been transformed already with bacterial genes encoding for the enzymes of GSH biosynthesis. Significantly increased GSH and γ-glutamylcysteine levels were found in plants transformed by genes encoding γ-ECS, which is the rate-limiting enzyme of GSH biosynthesis (Foyer and Rennenberg 2000, Noctor and Foyer 1998, Zhu et al. 1999). However, the effect of these transformations on the disease resistance has not been investigated yet. Obviously, the study of such transgenic plants will greatly aid in understanding the role of GSH in infected plants.

Artificial elevation of GSH content

An interesting theoretical possibility to increase plant disease resistance is the artificial elevation of the cellular GSH levels by biochemical methods. An early publication demonstrated that exogenously added ascorbic acid and GSH suppressed the symptoms of viral infections (Farkas et al. 1960).

Treatments of tomato and melon plants with dinitroaniline herbicides led to considerably increased endogenous GSH levels in the roots. These herbicide treatments induced resistance against the fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* and f. sp. *melonis.* In addition, significant correlations were observed between the GSH levels and the extent of protection against the fungal diseases. However, as the authors noted, the herbicide treatments brought about other metabolic changes in the roots, which might also lead to protection (Bolter et al. 1993).

Treatment of plants with various herbicide safeners (antidotes) can also bring about a marked increase in foliar GSH levels (Kömives 1992). The safener flurazol, which elevated the cellular GSH levels, significantly decreased the incidence of downy mildew infections in *Sorghum* plants (Kömives and Dutka 1989).

An interesting possibility for the elevation of foliar GSH levels is the use of the recently developed synthetic resistance inducer benzo-[1,2,3]-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH). BTH is capable of inducing several defence genes and of providing long-lasting protection to plants

against powdery mildew and other pathogens (Görlach et al. 1996). GSH levels of soybean cells were markedly increased by a 2-day-incubation with BTH and salicylic acid (Knörzer et al. 1999).

Recent data confirmed the protective role of elevated GSH levels in virus-infected tobacco leaves. Cellular GSH levels were markedly elevated in Xanthi-nc tobacco leaf discs by 2 mM L-2-oxothiazolidine-4-carboxylic acid (OTC). The synthetic L-cysteine precursor OTC has been reported earlier to substantially elevate GSH levels in plant tissues (Hausladen and Kunert 1990). The tobacco leaf discs having elevated GSH content were infected by TMV 2 days after the OTC pre-treatment. The artificial elevation of cellular GSH content markedly suppressed the development of necrotic disease symptoms and the number of necrotic lesions strongly decreased. In addition, the foliar virus concentration (as measured by ELISA) decreased in parallel with the lesion number, to 55 % of the control (Gullner et al. 1999). The mechanism, by which elevated GSH levels restrict TMV multiplication in tobacco leaf tissue are unknown. The ability of GSH to inhibit the replication of human viruses has already been shown (Palamara et al. 1995, Vossen et al. 1997). These experiments suggest that the investigation of early changes of GSH levels and GSH/GSSG ratios in virus-infected plants will define more precisely the role of GSH in the future.

BIOCHEMICAL FUNCTIONS OF GSH IN INFECTED PLANTS

Avirulent microbial pathogens (incompatible plant-microbe interactions) trigger the activation of a range of inducible defence responses in higher plants and as a result the damage caused by the pathogen remains restricted. One of the most important defence responses is the hypersensitive reaction (HR), which is usually characterized by the rapid appearance of necrotic spots containing dead cells at sites of attempted pathogen ingress. Necrotic lesions are clearly delimited from surrounding healthy tissue, and the localized host cell death contributes to pathogen limitation (Levine et al. 1994, Zhu et al. 1996). HR is often associated with the sustained production and accumulation of ROS (oxidative burst) (Lamb and Dixon 1997). Other important defence responses include the reinforcement of plant cell wall by deposition of lignin and hydroxyproline-rich glycoproteins, the induction of hydrolytic enzymes (such as chitinase and glucanase) and the biosynthesis of antimicrobial phytoalexins. This wide array of defence responses is brought about by specific interactions between elicitors originating from the pathogen and receptors of the host cell. The signal transduction pathways leading to the activation of defence reaction are only poorly understood (Hammond-

Kosack and Jones 1996, Guo et al. 1998). Numerous reports indicate, however, that GSH has important regulatory roles in almost all of the above listed defence reactions (Figure 1).

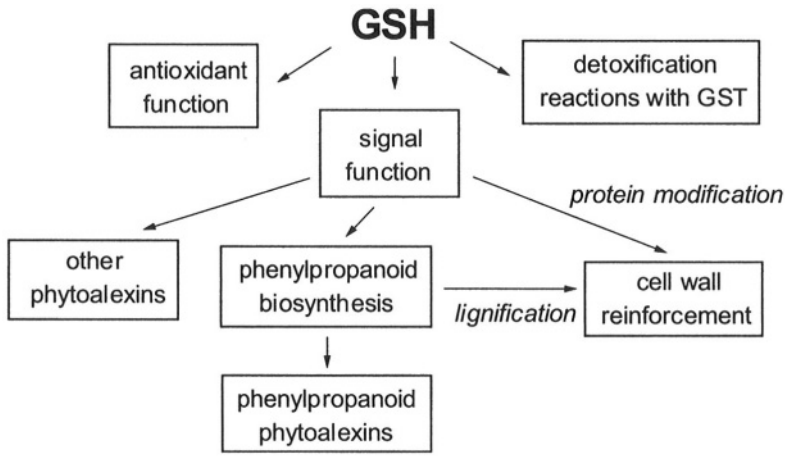

Figure 1. Functions of glutathione in plants infected by microbial pathogens.

Oxidative stress

The most rapid response of infected plants is the increased production and accumulation of ROS, which is known as oxidative burst or oxidative stress (Lamb and Dixon 1997). Based on analogies from animal studies it is supposed that ROS participate in the defence of the plant by directly killing the invading pathogen. On the other hand, oxidative stress leads to peroxidative damage of plant cell membranes (lipid peroxidation), cell death and the appearance of macroscopic necrotic lesions on the leaf surface. Lipid peroxidation and lesion formation are characteristic of HR. Hydrogen peroxide seems to play a central role in the oxidative burst: it acts as a signal for localized death of challenged cells (HR) and as a diffusible signal for induction of cellular protectant genes in adjacent cells (Levine et al. 1994).

One of the primary roles of GSH in plant tissues is the participation in antioxidative defence reactions. Plant cells have to avoid excessive oxidative damage in non-invaded tissues around pathogen entry sites in order to exert effective defence responses. Probably this is the reason for GSH accumulation in plant cells in response to an oxidative stimulus (May and Leaver 1993). GSH

can scavenge ROS in direct reactions (Winterbourn and Metodiewa 1999) or through the ascorbate-glutathione cycle, which is an important mechanism of plant cells for the detoxification of H_2O_2 (Foyer and Rennenberg 2000).

Superoxide dismutase isoenzymes (SODs, E.C. 1.15.1.1.) play an important role in the protection against ROS by decomposing superoxide radicals accumulating during oxidative stress (Lamb and Dixon 1997). Hérouart et al. (1993) have shown that the expression of a gene encoding Cu/Zn-SOD in *Nicotiana plumbaginifolia* was markedly induced by GSH and other reduced low molecular weight thiols (except for 2-mercaptoethanol), but not by GSSG. This is somewhat paradoxical, because in oxidative stress situations the steady-state concentrations of components of cellular redox systems are altered in favour of the oxidized forms. One would expect that GSSG, the oxidized component should induce antioxidative reactions. It seems that GSH can reduce a disulphide bridge, thereby activating a protein involved in reception or signal transduction of the oxidative stress response. Alternatively GSH may have a role in the activation of a transcription factor (Hérouart et al. 1993).

H_2O_2 production and changes in GSH content were followed in whole-leaf extracts from a susceptible and a resistant barley isolines between 12 and 24 hours after inoculation with *Blumeria graminis* f. sp. *hordei.* In hypersensitive response sites, H_2O_2 accumulation first occurred in the mesophyll underlying the attacked epidermal cell. Subsequently, H_2O_2 disappeared from the mesophyll and accumulated around attacked epidermal cells. In resistant plants, transient GSH oxidation coincided with H_2O_2 accumulation in the mesophyll. Subsequently, total foliar GSH + GSSG levels transiently increased in resistant tissues. These changes were absent from susceptible plants. It seems that an early intercellular signal precedes H_2O_2, and this elicits antioxidant responses in leaves prior to events leading to death of attacked cells (Vanacker et al. 2000). It is interesting to note that heat treatments of barley leaves resulted in the accumulation of ROS and induced resistance against inoculations with *B. graminis* f. sp. *hordei.* Heat-induced resistance was also associated with an increase of total glutathione levels (Vallélian-Bindschedler et al. 1998).

An interesting hypothesis was put forward suggesting that nicotinamide, and its metabolites nicotinic acid and N-methyl-nicotinic acid (trigonelline), act as signal mediating compounds, especially in association with stress release of nicotinamide from NAD following oxidative stress (Berglund 1994, Berglund and Ohlsson 1995). Nicotinamide was shown to increase GSH levels and to induce the phenylpropanoid/flavonoid metabolism (Berglund et al. 1993).

Reinforcement of plant cell wall

In addition to structural polysaccharides (including cellulose, pectin and hemicellulose polymers), plant cell walls contain also structural proteins: hydroxyproline-rich glycoproteins, proline-rich proteins and glycine-rich proteins (Bradley et al. 1992). The composition of the cell wall can be markedly altered by various environmental stimuli. The transcription of defence genes encoding cell wall hydroxyproline-rich glycoproteins was markedly stimulated by exogenous GSH in bean cell suspension (Wingate et al. 1988). These proteins were shown to be induced also by a fungal elicitor. Some years later it was shown that the treatment of bean or soybean cells with fungal elicitor or GSH led to a rapid insolubilization of pre-existing proline- and hydroxyproline-rich structural proteins in the cell wall (Bradley et al. 1992). This response to elicitor or GSH could be mimicked by exogenous H_2O_2 and inhibited by simultaneous addition of catalase or ascorbate. This rapid toughening process of cell walls, which involved H_2O_2-mediated cross-linking, was complete during the initial stages of plant defence reactions (within 10 minutes) and preceded the transcription of defence genes (maximum rates of transcription were observed after 1-3 hours). The insolubilization process should markedly increase the effectiveness of cell wall as a protective barrier to microbial ingress. H_2O_2 was putatively generated at the plasma membrane. The optimal GSH concentration for the insolubilization was 50 μM. By using 1 mM GSH this reaction is much slower which presumably reflects some H_2O_2 destruction as a competing reaction by the higher concentration of the reductant GSH (Bradley et al. 1992).

Phytoalexin formation

Phytoalexins are antimicrobial compounds that are synthesized by plants in response to microbial attacks and various abiotic stress effects (Kuc 1995). The modification of sulphydryl groups in plants has long been suspected to be involved in elicitation of phenylpropanoid defence responses. The accumulations of the phytoalexins medicarpin (Gustine 1981, 1987) and glyceollin (Stössel 1984) were induced by different sulphydryl-blocking reagents in clover callus and soybean seedlings, respectively. These effects could be reversed by D,L-dithiothreitol (DTT). DTT itself did not induce medicarpin biosynthesis in clover callus (Gustine 1987). Glyceollin synthesis was regulated by interaction of the blocking reagents with sulphydryl groups located mainly at the outer surface of the plasmalemma (Stössel 1984). These reports also suggested that the removal of endogenous GSH by

SH-reagents could interfere with the function of GSH in maintaining the reduced state of protein sulphydryl groups and this interference might result in the release of an endogenous elicitor for phytoalexin production. Some other reports are also known concerning the effects of thiol-blocking reagents on phytoalexin production (reviewed by Graham and Graham 1996).

Interestingly, pre-treatment of potato tuber tissues with sulphydryl-blocking reagents inhibited the rapid hypersensitive death of tuber cells following infection with an incompatible race of *Phytophthora infestans.* The hyphal growth and the fungal penetration were not inhibited by SH-reagents. In the case of a compatible race, however, the pre-treatment with SH-reagents did not change the infection process (Doke and Tomiyama 1978).

In 1988, GSH itself was found to induce activities of enzymes participating in phytoalexin biosynthesis. GSH markedly activated the transcription of genes encoding chalcone synthase (CHS, E.C. 2.3.1.74) and phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5) (Dron et al. 1988, Wingate et al. 1988). CHS participates in the formation of flavonoid pigments and isoflavonoid phytoalexins by catalyzing the first committed step in the biosynthesis of flavonoids (Hahlbrock and Scheel 1989). PAL is the key enzyme of phenylpropanoid biosynthetic pathway, which is involved in lignin and phytoalexin production (Hahlbrock and Grisebach 1979).

The massive stimulation of transcription of genes encoding CHS and PAL by GSH in bean suspension cultures was discovered by Wingate et al. (1988). The maximal accumulation of mRNAs encoding PAL and CHS was observed 6 hours after treatment with exogenous GSH (0.01 mM – 1 mM), and thereafter the amount of these transcripts declined. GSH induced also the accumulation of several other, unidentified polypeptides. The effect of GSH closely resembled that of a fungal elicitor prepared from *Colletotrichum lindemuthianum.* GSH caused a marked and prolonged increase in PAL enzyme activity. The oxidized form of glutathione (GSSG) was only a weak inducer of PAL activity, whereas the constituent amino acids of GSH or reducing agents were inactive (Wingate et al. 1988). The close resemblance between the effects of GSH and fungal elicitor does not necessarily imply the physiological role of GSH in elicitor action. Cell surface receptors can possess intramolecular disulphide bridges and GSH may be able to cleave such linkages and initiate gene activation without being involved in the signal transduction pathway (Malbon et al. 1987).

In addition to the above results, GSH-responsive *cis*-acting elements were identified on the promoter of a bean CHS gene in electroporated soybean protoplasts (Dron et al. 1988). The expression of a chimeric construct containing the promoter of the bean CHS 15 gene linked to a reporter gene was induced by GSH also in electroporated alfalfa protoplasts (Choudhary et al. 1990a). The activation of CHS genes in bean plants by GSH was accompa-

nied by structural changes in the chromatin associated with the proximal region of the promoter and these changes probably reflected the binding of transcription factors to *cis*-regulatory elements (Lawton et al. 1990).

In contrast to bean and soybean cells, alfalfa cells did not respond to GSH treatments without electroporation. Exposure to GSH did not change the PAL activity and did not lead to the production of the pterocarpan phytoalexin medicarpin in suspension cultured alfalfa cells (Dalkin et al. 1990). In addition, alfalfa protoplasts responded to fungal elicitor treatment by an increase of CHS activity but were unresponsive to GSH (Choudhary et al. 1990b). However, electroporation of protoplasts or the addition of polyethylene glycol resulted in strong responsiveness to GSH, suggesting that the slow rate of GSH uptake caused the lack of response in the untreated alfalfa protoplasts. The signal transduction pathways for elicitation by a macromolecular fungal elicitor and GSH were shown to differ both in terms of initial sites of action and in relation to subsequent events during gene activation (Choudhary et al. 1990b).

Several other reports showed the changes of phytoalexin levels in GSH-treated plants. Exposure to 50 mM GSH led to the considerable accumulation of the pterocarpan phytoalexin, pisatin, in pea epicotyls 12 hours after treatment, with a maximum effect after 42 hours (Yamada et al. 1989). In addition, GSH enhanced the effect of chitosan on coumarin biosynthesis in parsley cell culture (Conrath et al. 1989). When root cultures of transgenic *Lotus corniculatus* were treated with exogenous GSH (2-10 mM), isoflavan phytoalexins (vestitol and sativan) accumulated in both tissue and culture medium. This phytoalexin accumulation was preceded by a transient increase in PAL activity (Robbins et al. 1991). Optimal GSH concentrations were found in the range of 1-4 mM to elicit PAL induction. PAL activities peaked 8 hours after the addition of GSH (2 mM) after a lag phase of approx. 3 hours (Robbins et al. 1991). Treatment with a fungal elicitor resulted in more rapid initial accumulation of vestitol when compared with GSH, however sativan (the 2-methoxy ester of vestitol) was only detected following elicitation with GSH. Both the elicitor and GSH also induced the accumulation of other phenylpropanoid compounds putatively identified as chalcones (Robbins et al. 1995).

From to the above results, it was suggested that GSH may elicit signal transduction processes leading to the induction of defence reactions following infection. However, Edwards et al. (1991), suggested that changes of endogenous GSH level are the consequences rather than the cause of the elicitation signal. They found that both GSH and GSSG elicited the phytoalexin response (isoflavonoid biosynthesis) in bean cell suspension cultures but not in those of alfalfa. The exposure of bean cells to a fungal elicitor resulted in the accumulation of hGSH but not GSH. Treatment of alfalfa

cells with a fungal elicitor resulted in the increase of endogenous GSH levels. However, treatments of bean or alfalfa cells with the synthetic cysteine precursor OTC, which also increased the intracellular thiol level did not elicit phytoalexin biosynthesis. In addition, the changes in tripeptide thiol levels occurred too slowly to be consistent with a role in the initiation of the elicitation response. The activation of PAL transciption occurs within minutes in elicitor treated bean cells whereas changes in thiol GSH or hGSH levels occur over hours. Based on these observations, it was supposed that the increased levels of GSH or hGSH in an elicited cell could protect the cell from oxidative damage (Edwards et al. 1991). In accordance with these results, OTC treatment of pea seedlings did not induce PAL activity and did not elicit the accumulation of the phytoalexin pisatin in leaf tissues, except for high, phytotoxic OTC concentrations. These observations confirmed that a stress response regulatory mechanism based on GSH concentrations above normal levels is less probable (Kömives et al. 1997). Lack of antioxidant gene activation after OTC treatment was observed by Hausladen and Kunert (1990). It is possible, however, that transient changes in GSSG/GSH ratios or redistribution of thiols in various internal pools might act as signals for elicitation in bean cells in response to exogenously applied GSH. Protein activation might result also from S-thiolation processes.

The artificial decrease of cellular GSH levels in plants can also bring about phytoalexin accumulation. A dramatic reduction of GSH levels (down to 5 % of control) was achieved in suspension-cultured carrot cell cultures by L-buthionine-[S,R]-sulphoximine (BSO), which is an inhibitor of GSH biosynthesis. In addition, BSO treatments led to markedly increased levels of both H_2O_2 and the carrot phytoalexin 6-methoxymellein. The authors supposed that active oxygen species accumulated due to the GSH depletion and this triggered the phytoalexin synthesis. Exogenously added GSH suppressed the phytoalexin accumulation in the BSO-treated cells (Guo et al. 1993). The effect of BSO on the phytoalexin production was synergistically enhanced by the co-treatment of carrot cells with a yeast glucan elicitor (Guo and Ohta 1993). In accordance with these results, treatment of soybean seedlings with sulphydryl reagents and hydroperoxides led to the accumulation of the phytoalexin glyceollin. It was suggested that lipid hydroperoxides that accumulated in all treatments are involved in the signal cascade leading to elicitation. However, several hydroperoxide treatments leading to elicitation did not change the concentration and redox state of the intracellular non-protein thiol pool (predominantly hGSH). These results showed that an immediate decrease of the free hGSH level is not necessary but could be sufficient for signal transduction (Degousée et al. 1994).

GSH showed significant and consistent effects on the various soluble phenylpropanoid or phenolic polymer responses in soybean cotyledon tis-

sues (Graham and Graham 1996). The responses of soybean initiated by an elicitor prepared from *Phytophthora sojae* include the accumulation of daidzein, the first committed precursor of the phytoalexin glyceollin. In the presence of this elicitor, GSH strongly enhanced the accumulation of coumestrol, but had very little net effect on daidzein, genistein or glyceollin accumulation. GSH alone markedly induced the formation of malonylglucosyl genistein. In addition, there is a specific interaction between GSH and the wound stimulated accumulation of phenolic polymers. When wound-associated elicitor(s) are present, GSH greatly enhances the deposition of phenolic polymers into the cell wall. This response includes the peroxidase-mediated deposition of lignin- and suberin-like polymers. In this soybean system GSH did not seem to function as a primary elicitor, but it specifically and markedly enhanced the responses of wounded and elicitor-treated tissues (Graham and Graham 1996).

Recently a cDNA encoding a novel DNA-binding protein, which binds to the promoter of the CHS gene *chs15*, was isolated from soybean. This DNA-binding protein was rapidly phosphorylated in soybean cells during the elicitation of phenylpropanoid biosynthetic genes, almost exclusively on serine residues. A cytosolic protein-serine kinase was shown to phosphorylate the DNA-binding protein. This kinase enzyme was rapidly and transiently stimulated in cells elicited with either GSH or an avirulent strain of the soybean pathogen *Pseudomonas syringae* pv. *glycinea.* Phosphorylation of the DNA-binding protein *in vitro* enhanced binding to the *chs15* promoter. It seems that stimulation of the serine kinase activity and phosphorylation are terminal events in a signal pathway for activation of early transcription-dependent plant defence responses (Dröge-Laser et al. 1997).

The above results showed that phytoalexin accumulation can be induced by both decreasing and increasing GSH levels, and by sulphydryl-blocking reagents (Figure 2). In spite of numerous studies, the role of GSH in the elicitation of phytoalexin production has remained elusive. It seems that the disturbance of the cellular redox balance, which is influenced also by the GSSG/GSH redox couple, may lead to phytoalexin accumulation. More detailed analysis of early events of elicitation and of alterations of the cellular redox state are necessary to assess more precisely the regulatory roles of GSH in phytoalexin biosynthesis.

Primary metabolism and GSH

Beside the production of secondary plant metabolites such as phytoalexins and lignin, GSH can significantly influence also the primary metabolism that provides building blocks and energy for the biosynthesis of defence

compounds. Malate is involved in a wide variety of primary physiological processes. Malate oxidation is accomplished through various enzymes including the NADP-dependent malic enzyme (E.C. 1.1.1.40.), which catalyses the oxidative decarboxylation of malate to pyruvate, producing CO_2 and NADPH. A promoter fragment of the gene encoding the malic enzyme was prepared from bean and fused to a gene encoding β-glucuronidase (GUS reporter gene). Tobacco plants were transformed by this chimeric gene construct and the regulatory properties of the promoter sequence were studied by detecting the histochemical staining of GUS activity. GSH (10 mM) strongly induced the GUS activity, *i.e.* the transcription from the promoter of malic enzyme, but GSSG, ascorbic acid, and a fungal elicitor were also effective promoter-stimulating agents (Schaaf et al. 1995). By the activation of malic enzyme the production of reducing equivalents (NADPH) can be increased. The rapid access to NADPH is of crucial importance for antioxidative defence reactions *e.g.* for the reduction of GSSG by GR enzyme, for phytoalexin biosynthesis, and for the reductase activity of a disease-resistance gene (Schaaf et al. 1995). Significantly increased NADPH-consuming activity was found in leaf cell-free extracts of a highly susceptible barley cultivar following inoculation with *Blumeria graminis* f. sp. *hordei* (El-Zahaby et al. 1995).

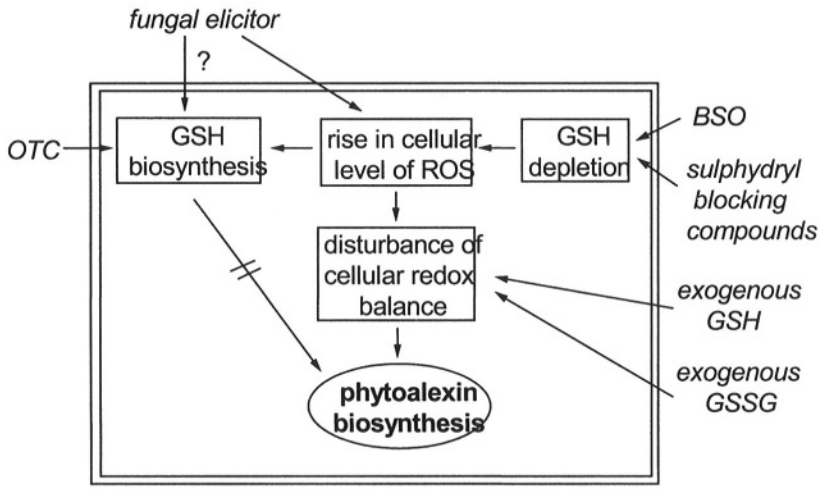

Figure 2. Schematic representation of glutathione-related signal transduction pathways leading to the activation of phytoalexin biosynthesis in a plant cell (shown as a double lined box) following different external stimuli. Abbreviations: BSO, L-buthionine-[S,R]-sulphoximine; GSH, glutathione, GSSG, oxidized glutathione; OTC, L-2-oxothiazolidine-4-carboxylic acid; ROS, reactive oxygen species.

Oxidative stress is thought to alter the cellular redox equilibrium toward a more oxidized status. Therefore, it is surprising that antioxidative substances such as GSH and ascorbic acid led to powerful inductions of the promoter of malic enzyme, while GSSG was less efficient and exogenous H_2O_2 had no stimulating effect (Schaaf et al. 1995). It is supposed that GSH acted directly as an antioxidant and simultaneously activated a panoply of stress genes (Hérouart et al. 1993, Schaaf et al. 1995).

GLUTATHIONE-RELATED ENZYMES AND PATHOGENESIS

Glutathione *S*-transferases in fungal infections

Among the enzymes related to GSH metabolism, the role of glutathione *S*-transferase (GST, E.C. 2.5.1.18) isoenzymes has been the most extensively studied in infected plants. This isoenzyme family has been known since 1970 to have a significant and well-defined role in plant detoxification reactions (Marrs 1996, Edwards et al. 2000). The GST enzymes are homo- or heterodimeric combinations of different subunits. GSTs catalyse the binding of various xenobiotics (including numerous pesticides) and their electrophilic metabolites with GSH to produce less toxic and more water-soluble conjugates (Edwards et al. 2000). Besides catalyzing the conjugation of electrophilic compounds to GSH, GST isoenzymes also exhibit peroxidase activity (Bartling et al. 1993) (Figure 3). Various abiotic stress effects are powerful inducers of GST activity in plants (Dixon et al. 1998).

$$\text{a)}\quad \text{R-X} + \text{GSH} \xrightarrow{\text{GST}} \text{R-SG} + \text{X-H}$$

$$\text{b)}\quad \text{R-OOH} + 2\,\text{GSH} \xrightarrow{\text{GST}} \text{R-OH} + \text{GSSG} + \text{H}_2\text{O}$$

Figure 3. The most important reactions catalysed by glutathione *S*-transferase enzymes in plant cells: a) detoxification of various xenobiotics (R-X) in conjugation reactions, b) decomposition of lipid hydroperoxides (R-OOH) (glutathione peroxidase function).

The first evidence suggesting the participation of GSTs in plant-pathogen interactions came from studies on the interaction of wheat with a non-pathogen fungus in 1991. Wheat plants infected with the non-pathogen *Erysiphe graminis* f. sp. *hordei* (barley powdery mildew) showed local, induced

resistance against a second infection with the pathogen *Erysiphe graminis* f. sp. *tritici.* One of the genes activated simultaneously with the onset of resistance was shown to encode a GST isoenzyme (Dudler et al. 1991). Transcripts of this *GstA1* gene were at least 20 times more abundant in the leaves 14 hours after infection with *E. graminis* f. sp. *hordei* than in control leaves. In contrast, 48 hours after infection the GST enzyme activity was only 2-fold higher in the infected leaves as compared to control ones. It was supposed that the role of GST in infected plants may be the protection of plant cells against the toxic products of membrane lipid peroxidation. GST genes can also be the members of a class of general stress response genes, which are activated by many different stimuli (Dudler et al. 1991). From wheat a second, presumably defective GST gene (*gstA2*) was also isolated and sequenced which contained transposon-like sequences in the promoter region (Mauch et al. 1991).

In the same year the induction of GST activity in cultured French bean cells by a fungal elicitor was also reported. The GST activity (measured with cinnamic acid as substrate) was increased two- to three-fold at 4-8 hours after exposure of the cells to the elicitor prepared from the cell walls of the bean pathogen *Colletotrichum lindemuthianum.* No induction of the activity was found when the generally used l-chloro-2,4-dinitrobenzene was utilized as substrate. The proposed role for the GST was the detoxification of olefinic substrates formed as activated intermediates during the elicitation response (Edwards and Dixon 1991). However, recent work has shown that ascorbate peroxidase catalyses the addition of GSH to olefinic bonds by indirectly generating thiyl free radicals of GSH which can react with the alkyl double bond of cinnamic acid (Dean and Devarenne 1997).

Some years later the wheat *GstA1* gene was shown to encode a 29 kD GST (GST29) protein. This gene was specifically inducible by fungal infections and GSH, but not by various xenobiotics (Mauch and Dudler 1993). The *GstA1* gene was only weakly expressed in uninfected plants. Within 2 h after infection with *E. g.* f. sp. *hordei* or *E. g.* f. sp. *tritici*, the *GstA1* mRNA level increased dramatically and remained elevated for 2 days. The level and time course of expression of *GstA1* were similar in the incompatible and compatible interactions. The induction started well before the penetration of the host cell that begins about 6-10 hours after inoculation. Compared with uninoculated seedlings, GST enzyme activity was increased by a factor of only 1.2 in wheat plants 48 hours after inoculation. Probably other GST isoenzymes having high GST activity were masking the induction of GST29. Inoculation with another fungal pathogen, *Puccinia recondita* f. sp. *tritici* also induced the accumulation of *GstA1* mRNA. In contrast to GST29, other wheat GST isoenzymes had higher basal activity and their expressions were strongly induced by herbicides and other xenobiotics, but not by pathogen

attack. The specific induction of GST29 by pathogens implies that the enzyme has specific functions related to pathogen attacks. The detoxification of products originating from lipid peroxidation by GST29 may prevent continued cell disruption caused by highly toxic radicals and thereby localize the host response as seen in the HR (Mauch and Dudler 1993).

Infection of potato with the late blight fungus *Phytophthora infestans* results in the activation of various defence-related genes. Accumulation of mRNAs deriving from the *prp1* gene family is selectively induced by pathogen attack and not in response to abiotic stimuli. One member of this gene family (*prp1-1*) was shown to encode a cytosolic GST enzyme. The PRP1-1 mRNA and protein were detectable in the potato leaves at 2 and 6 hours post-inoculation, respectively. Both mRNA and protein reached maximum concentrations between 48-56 hours after infection and the concentrations remained at high levels throughout the infection cycle (massive sporulation started 6 day post-inoculation in the compatible interaction). No qualitative differences in the time courses of PRP1-1 mRNA and protein accumulations were observed when compatible and incompatible interactions were compared. The higher absolute amount of mRNA and protein reached in compatible interactions are possibly due to the larger number of host cells affected in this type of interactions. Similarly to the observations of Mauch and Dudler (1993), only slight changes of GST enzyme activity were found in the infected leaves, possibly due to high constitutive levels of other GST isoenzymes. The authors supposed that GST-dependent detoxification processes are involved in controlling the extent of hypersensitive cell death at infection sites (Hahn and Strittmatter 1994).

GST activities were determined in leaves of three barley cultivars following inoculations with *B. graminis* f. sp. *hordei.* GST was markedly induced in each mildewed barley cultivar, but the rate and timing of the induction were different among the cultivars. The most substantial rate of induction was found in a highly susceptible cultivar. The enzyme activity increased gradually and 6 days after infection it reached 360 % of uninfected control. The rate and extent of induction was smaller in a moderately susceptible and in a resistant cultivar. Beside GST induction other antioxidative processes were also activated in the compatible host-parasite relationship (El-Zahaby et al. 1995).

GST in viral infections

The first observations on alterations of foliar GST activities in virus infected plants were made in various *Sorghum* - sugarcane mosaic virus (SCMV) interactions. Three *Sorghum* varieties expressing different suscep-

tibility towards SCMV were inoculated by the MB strain of the virus. Markedly increased GST activities were found in an immune-type *Sorghum* host after inoculation (incompatible interaction). In contrast, viral infections led to strongly decreased GST activities in a moderately susceptible and in a susceptible cultivar (compatible interactions). The mechanisms leading to the repression of GST activity are not known (Gullner et al. 1995a). Diminished GST activity can enhance cellular sensitivity to a wide range of toxic metabolites produced during pathogen infection and these conditions may be favourable for virus multiplication. Markedly decreased GST activities were found also in virus-infected animal and human cell lines (Jaitovitch-Groisman et al. 2000). In these cells the GST expression was transcriptionally regulated.

As mentioned earlier, the potato defence gene *gst1* (formerly called *prp1-1*) encodes a GST isoenzyme (Hahn and Strittmatter 1994). Promoter fragments of this *gst1* gene were fused to a bacterial *gus* gene in order to detect the transcription regulated by these short promoter fragments with histochemical GUS staining. Potato plants were transformed with these chimeric gene constructs, and the transgenic potato lines were exposed to viral inoculations and other stress effects. Transcription from the promoter fragments was not induced by abiotic stimuli, such as wounding or heat shock. Transgenic potato plants were inoculated with two different strains of the pathogenic potato virus Y (PVY). The common O strain, PVY^O caused severe necrosis, whereas the veinal necrotic strain, PVY^N induced mild symptoms characterized by a vague mottling on leaves. Inoculation by both strains resulted in the formation of blue spots indicating GUS expression after 3 days. The pattern of GUS activity paralleled the spread of PVY in the leaves (Strittmatter et al. 1996). The responsiveness of the *gst1* promoter fragment to inoculation with potato leaf roll virus (PLRV) was also investigated. Infections with PLRV resulted in leaf rolling and chlorosis. Leaves of plants showing clear symptoms of virus infection were histochemically assayed for GUS activity. Blue staining was restricted to the vascular system of leaves, indicating that the activity of the *gst1* promoter fragment was strictly limited to the tissue, in which the virus multiplied (Strittmatter et al. 1996).

Markedly induced foliar GST activities were found also in other incompatible interactions. Tobacco mosaic virus (TMV) infection led to substantial induction of GST activity (preceded by a slight, transient decline) both locally and systemically in infected *Nicotiana tabacum* L *cv.* Xanthi-nc plants (Fodor et al. 1997). Leaves of this resistant tobacco cultivar react hypersensitively to TMV inoculation. TMV brings about an oxidative stress leading to lipid peroxidation, membrane destruction, cell death and the appearance of necrotic lesions (HR) (Doke and Ohashi 1988, Gullner et al. 1997). The elevated GST activities observed in TMV-inoculated plants suggest that there is an in-

creased demand for detoxification reactions. Detoxification reactions catalysed by GST can participate in the suppression of necrotic reactions and lesion formation (Fodor et al. 1997). Tobacco necrosis virus (TNV) inoculation also led to elevated GST activities in *Nicotiana tabacum* L. cv. Samsun (Gullner et al. 1995b).

The volatile monoterpene (S)-carvone was found to induce massively the GST activity in tobacco leaf discs up to 9-12-fold of control (Gullner et al. 1999). Beside GST, other antioxidative enzymes were also induced, but to a much less extent. When carvone-pretreated leaf discs were inoculated with TMV, the carvone pre-treatment strongly reduced the number and size of necrotic lesions. However, carvone pre-treatments did not influence the virus concentration, *i.e.* the lesion formation and virus multiplication did not correlate. The marked induction of GST activity may be necessary for the detoxification of carvone. On the other hand, the lipid peroxidation observed in carvone-treated leaves showed that the induction of GST might defend plant cells against the deleterious consequences of oxidative stress. The elevated capacity of carvone-treated leaf tissue to detoxify lipid hydroperoxides by GST probably contributed to the suppression of necrotic symptoms, but it did not affect the virus multiplication (Gullner et al. 1999).

Generally, marked GST inductions were observed in incompatible plant - virus interactions. The exact role of GST induction in this interaction is not known. Further comparisons of other incompatible and compatible interactions are necessary for a better understanding of the role of GSTs in virus infected plants.

GST in bacterial infections

Bacterial infections can cause also oxidative stress leading to the formation of lipid hydroperoxides (Croft et al. 1993). The induction of GST activity was reported in *Arabidopsis* and bean plants inoculated with the pathogen *Pseudomonas syringae* pv. *phaseolicola* (Greenberg et al. 1994, Ádám et al. 1997). Markedly induced GST activities were found in bean leaf tissues surrounding HR lesions after inoculation with the incompatible race 3 of *P. syringae* pv. *phaseolicola.* GST induction was preceded by a decrease in catalase activity. In leaves infected by the compatible race 6 of *P. syringae* pv. *phaseolicola,* no significant GST induction was found. It was supposed that declining catalase activity caused limited oxidative stress in tissues surrounding the HR lesions, and GST detoxified toxic products of lipid peroxidation (Ádám et al. 1997).

The accumulation of mRNAs encoded by a GST gene and by two other, distinct defence-related genes was observed in *Arabidopsis thaliana* leaves inoculated by the bacterial pathogen *Pseudomonas syringae* pv. *tomato.* This bacterium can produce the phytotoxin coronatine, which markedly contributes to the development of disease symptoms (lesion expansion, chlorosis formation). Inoculations with a coronatine-deficient mutant bacterium caused only mild disease symptoms, but consistently induced higher levels of GST transcripts than the coronatine-producing strain. The accumulation of GST mRNA was 2- to 5-times more abundant in plants inoculated with the coronatine-deficient strain than those induced by the coronatine-producing strain. Coronatine may play a critical role during early stages of infection by suppressing the activation of defence-related genes (Mittal and Davis 1995).

The role of GST was investigated also in pear and tobacco plants infected with *Erwinia amylovora,* the causal agent of fire blight disease. The bacterium caused a sustained oxidative stress and GST induction in both pear and tobacco leaves (in compatible and incompatible interactions, respectively). The unexpected ability of *E. amylovora* to generate oxidative stress even in a compatible situation was linked to its functional *hrp* gene cluster. It was suggested that *E. amylovora* utilizes the production of ROS as a tool to provoke host cell death to invade plant tissues (Venisse et al. 2001).

GST as oxidative stress marker

Hydrogen peroxide generated during an oxidative stress has a dual role in infected plants. It can act as a trigger for localized cell death and also as a rapid, inductive signal for expression of antioxidative defences. The induction of cellular protectant genes occurs at lower doses of H_2O_2 than required for HR, and the induction takes place in healthy cells adjacent to necrotized spots in infected leaves. The accumulation of H_2O_2 in soybean cell suspension cultures was activated by a fungal elicitor prepared from *Phytophthora megasperma* f.sp. *glycinea* or by the avirulent bacterial pathogen *Pseudomonas syringae* pv. *glycinea.* The signal transduction pathway involved a protein Ser/Thr kinase cascade that leads to the activation of the plasma membrane associated NADPH oxidase complex, which is supposed to be the main source of ROS production. As a consequence of oxidative burst, the accumulation of mRNA encoding GST was observed 1 hr after elicitation with the fungal elicitor and 8 hours after bacterial infection. Inoculation with a virulent strain of *P. syringae* pv. *glycinea* did not result in GST transcript accumulation (Levine et al. 1994).

Following this report, several papers have reported the induction of GST transcripts as a consequence of oxidative stress resulting from different plant-pathogen and plant-elicitor interactions. Treatment of soybean cell suspension cultures with an avirulent race of *Pseudomonas syringae* pv. *glycinea* or with yeast elicitor resulted in oxidative stress and in the induction of GST transcripts. This induction occurred more rapidly and was more prolonged if cells were simultaneously treated with serine protease inhibitors. These results revealed the involvement of a protease inhibitor-sensitive, negative regulatory step in the signal transduction pathway leading to both oxidative stress and phytoalexin accumulation (Guo et al. 1998). Inoculation of *Arabidopsis thaliana* with an avirulent strain of *Pseudomonas syringae* pv. tomato resulted in oxidative burst and the accumulation of mRNA encoding GST, not only in the inoculated leaves, but also systemically *i.e.* in the uninfected, secondary leaves. The transcript accumulation in the secondary leaves occurred between 1 and 6 hours after inoculation, slightly delayed relative to the inoculated (primary) leaves. No such accumulation was found in plants inoculated with a virulent strain (Alvarez et al. 1998).

Role of GST activity in infected plants

A major obstacle in understanding the role of GSTs in infected plants is the fact that the endogenous, physiological substrates of GST isoenzymes are in most cases not known. Among the possible substrates are fatty acid hydroperoxides, which are major products of oxidative membrane damage and can be also produced by lipoxygenase action. Bartling et al. (1993) isolated a cDNA clone for a novel GST from *Arabidopsis.* The encoded protein catalysed the decomposition of several fatty acid hydroperoxides into the corresponding hydroxy-acids, with the concomitant formation of GSSG (Figure 4). However, the enzyme did not use H_2O_2 as substrate. These data confirmed that GSTs contribute to the protection against oxidative damage to membranes in plants.

Ethacrynic acid, which resembles naturally occurring alkenals accumulating during oxidative stress (Croft et al. 1993), was used as a model GST substrate (Edwards 1996). Exposure of pea epicotyls to a fungal cell wall elicitor or GSH markedly stimulated the GST activity toward ethacrynic acid, which was not detectable in water-treated controls. It is important to note that elicitations did not increase GST activities towards the generally used xenobiotic substrates 1-chloro-2,4-dinitrobenzene or fluorodifen. Fungal elicitor and GSH treatments resulted also in the accumulation of the phytoalexin pisatin. It is possible that elicitor-induced GSTs function to conju-

gate toxic alkenals, which might accumulate in plants during oxidative stress (Edwards 1996).

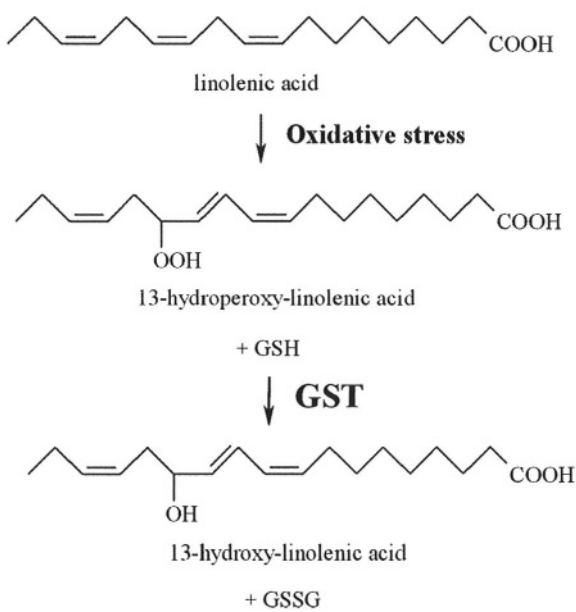

Figure 4. Possible role of glutathione *S*-transferase (GST) isoenzymes in infected plants: detoxification of fatty acid hydroperoxides by the peroxidase activity of GST as described by Bartling et al. (1993). The formation of 13(S)-hydroperoxy-9,11,15-octadecatrienoic acid (13-hydroperoxy-linolenic acid) is a typical example of the oxidation of unsaturated fatty acids during membrane-damaging lipid peroxidation processes. GST can catalyse the detoxification (reduction) of lipid hydroperoxides into the corresponding hydroxy derivatives.

The artificial elevation of GST in plant tissues can provide valuable information on the role of GST in infected plants. Recently a substantial induction of GST activity was observed in Xanthi-nc tobacco leaf discs treated with the monoterpene compound (S)-carvone. Tobacco leaf discs with induced GST activities were infected by TMV 2 days after (S)-carvone pretreatment. The induction of GST activity markedly suppressed the development of necrotic lesions induced by TMV, but did not decrease the foliar virus concentration. These results confirmed previous suppositions about the protective role of GST in virus-infected tobacco leaves (Gullner et al. 1999). GST activities of soybean cells were markedly increased by 2 days incubations with BTH and salicylic acid, but the resistance of the treated cell cultures toward infections was not investigated (Knörzer et al. 1999).

Summarizing the above reports on GST, it seems that the role of GSTs in infected plants is the suppression of necrotic disease symptoms by the detoxification of toxic lipid hydroperoxides that derive from peroxidation of cell membranes. In the future, this supposition may be confirmed by the study of transgenic plants overexpressing GST isoenzymes and of transformants expressing GSTs in antisense direction. Tobacco plants have been transformed with genes encoding GST (Roxas et al. 1997, Thompson et al. 1998). These transgenic tobacco plants overexpressing GST were more resistant towards abiotic stress effects than wild type plants. The effects of microbial infections on these transgenic plants have not been reported yet.

Glutathione reductase

Little information is available about pathogen-induced changes in the antioxidant GR enzyme activities. GR activities were measured in leaves of two barley varieties, a resistant line showing hypersensitive race-specific resistance to avirulent races of *Blumeria graminis* and a susceptible line. In non-inoculated whole leaf extracts GR activity in the resistant leaves was double that of susceptible leaves. Inoculation with *B. graminis* f. sp. *hordei* resulted in a substantial decrease of GR activity in the resistant line, whereas no significant change was found in susceptible plants (Vanacker et al. 1998a). Similar results were found in oat (*Avena sativa* L.) lines, which expressed different degrees of susceptibility towards *B. graminis* f. sp. *avenae.* Inoculation caused a substantial decrease in foliar and apoplastic GR activities of a resistant line but not in more susceptible cultivars (Vanacker et al. 1998b). In contrast to these results, El-Zahaby et al. (1995) found no significant alterations of GR activity in three barley cultivars following *B. graminis* inoculation.

Inoculation of tomato leaves with the necrotrophic fungal pathogen *Botrytis cinerea* led to markedly increased GR activities. The induction of GR activity coincided with the appearance of visible disease symptoms. GR activity was sufficient to prevent the accumulation of GSSG and to maintain high and constant GSH/GSSG ratio in the infected leaves (Kuzniak and Sklodowska 1999).

Markedly altered GR activities were found in TMV-inoculated *Nicotiana tabacum* L. *cv.* Xanthi-nc leaves. Symptom appearance after TMV inoculation was preceded by a slight, transient decline of GR activities in the inoculated leaves, but after the onset of necrosis the GR activity substantially increased reaching 175 % of control. GR activities were induced not only in infected leaves, but also systemically, 10-14 days post TMV-inoculation concomitantly with the development of SAR. Injection of salicylic acid (0.8 mM) into tobacco

leaves also induced the GR activities (Fodor et al. 1997). The elevated GR activities confers a more efficient antioxidative protection to infected tissues. Interestingly, the synthetic resistance inducer D,L-β-aminobutyric acid brought about a marked induction of GR activity in pea and tobacco leaf discs, but did not induce several other antioxidative enzymes. It was supposed that elevated GR activity can contribute to disease resistance (Gullner and Sirály 1996).

Glutathione peroxidases

Glutathione peroxidases (GPOX, E.C. 1.11.1.9) are a family of isoenzymes, which catalyse the reduction of H_2O_2 and organic hydroperoxides including lipid- and phospholipid hydroperoxides by using GSH. These isoenzymes help to protect cells against oxidative damage. In recent years, indications for the existence of GPOX isoenzymes in plants have been reported (Edwards 1996, Eshdat et al. 1997, Dixon et al. 1998). Genes encoding stress-inducible phospholipid hydroperoxide GPOXs were cloned from several plant species (Sugimoto and Sakamoto 1997, Dixon et al. 1998). It must be noted that certain GSTs have also secondary GPOX activities and are able to reduce lipid hydroperoxides to their corresponding less toxic alcohols (Bartling et al. 1993). However, these GSTs showed no activity toward phospholipid hydroperoxides and H_2O_2 (Dixon et al. 1998).

Genes encoding homologues of mammalian GPOXs were isolated from tobacco. The putative GPOX gene was highly expressed in leaves exposed to $HgCl_2$ and infected by green tomato atypical mosaic virus, which caused the formation of local lesions on tobacco leaves (Criqui et al. 1992). The cloning of two GPOX-encoding cDNA sequences isolated from sunflower has been recently reported. The infection of sunflower plants by the biotropic fungus *Plasmopara halstedii* causing downy mildew led to a marked accumulation of GPOX transcripts. Sequence alignments suggested that these GPOX sequences are related to animal phospholipid hydroperoxide GPOXs (Roeckel-Drevet et al. 1998). The function of these stress-inducible GPOXs is probably the protection of biomembranes against oxidative damage.

CONCLUSIONS AND FUTURE PERSPECTIVES

The concept that GSH is one of the regulating chemicals in defence reactions of plants during plant-pathogen interactions is now generally accepted. However, no clear picture has emerged yet about the exact roles of GSH in infected plants. These roles are very difficult to identify because of the multi-

plicity and interdependence of GSH-related biochemical pathways. Obviously, GSH plays a crucial role in the removal of toxic metabolites after pathogen attacks and can protect intact plant cell surrounding infected cells from oxidative damage. Plants seem to react very sensitively to redox perturbations. Changes in GSH concentrations are able to affect the activities of various proteinaceous components of signal transduction pathways by thiol/disulfide redox reactions. GSH-dependent reactions can markedly influence the accumulation of the signal molecule H_2O_2 (Foyer et al. 1997). It is possible that alterations in the GSSG/GSH ratio are more decisive in determining plant resistance than changes in the GSH level. Further investigations of cellular thiol/disulphide redox reactions will undoubtedly help to unravel the complexity of GSH-dependent protective reactions.

Several aspects of GSH metabolism have not been investigated in plant-pathogen interactions. An interesting topic for future research may be the possible regulatory role of protein-bound GSH (reversible *S*-glutathionylation of proteins) in plant defence reactions. The significance of such processes has been demonstrated in the desiccation tolerance of plants (Kranner and Grill 1996), but their role in infected plants is unknown. In addition, apparently no information is available about the effects of microbial infections on the enzymes of GSH biosynthesis in plants. It is known that increases in GSH levels that occur in response to various abiotic stress effects result from post-translational activation of γ-ECS, the rate-limiting enzyme of GSH biosynthesis. Discrete signal transduction pathways exist that permit discrimination between different stresses and that activate appropriate responses (May et al. 1998b). However, the potential activation of γ-ECS in infected plants has not been investigated. It is conceivable that the enzymes of assimilatory sulphate reduction pathways are up-regulated in infected plants. Recently developed fluorescent labelling methods for the histochemical detection of GSH (Fricker et al. 2000, Meyer and Fricker 2000) will probably become a versatile tool to detect changes of GSH levels in infected individual cells and in cell compartments. Accumulating information about promoter sequences of GST genes, and about the regulation of GST transcription will also provide useful information. It is known that distinct GST isoenzymes are differentially induced by abiotic and biotic stress effects (Mauch and Dudler 1993, Pascal et al. 2000), but only very limited information has been gathered on the induction of distinct GST isoenzymes in pathogen-infected plants. Molecular biological methods, including the study of transgenic plants overexpressing GSH-related enzymes, are likely to provide useful information on the role of GSH in plant disease resistance in the near future.

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Chapter 10

ASPECTS OF GLUTATHIONE IN THE INTERACTION BETWEEN PLANTS AND ANIMALS

Ewald Schnug and Christine Sator

INTRODUCTION

Plants cannot move and cannot talk but still they interact with other organisms in their environment. They may be welcomed as pollinators or approach hostile as predators, the only tools plants have to interact with other organisms are structural or chemical. There is a broad range of chemicals in the plant kingdom with a known or supposed ecophysiological action. Sulphur (S) containing compounds are well known for their ecophysiological significance (Schnug 1993, 1997). Investigations on "Sulphur Induced Resistance" (SIR) is a new field of research whose objective is to employ improved knowledges in plant physiology. Thus new production methods in which pesticides are replaced by physiological "know-how" will be established (Schnug 1997). Volatile sulphur compounds, sulphur-rich storage proteins, phytochelatins, glucosinolates and phytoalexins, for example, have already been identified as substances with phytopathological potential (Schnug 1990, 1997).

The thiol tripeptide glutathione (GSH) in plant metabolism either as a structural compound or as a vital redox-system is subject of many research efforts and the knowledge in this field increases steadily (Rennenberg et al. 2000, Foyer et al. 2000, Davidian 1999). Glutathione has been found to be involved in different sorts of abiotic (Grill et al. 1986, 1989) and biotic stress factors (viruses, bacteria and fungi, e.g. Gullner et al. 1995a,b, May et al. 1996a,b).

However, less is known about the ecophysiological significance of GSH in the interaction of plants with animals, to which the topic of this contribution is dedicated.

D. Grill et al. (eds.), Significance of Glutathione to Plant Adaptation to the Environment, 241-248.

DIRECT INTERACTIONS BETWEEN PLANT GLUTATHIONE AND ANIMALS

The following physiological mechanisms involving GSH are called "direct interactions" as there is a substantial involvement of the compound GSH itself.

Glutathione affects the hatching of parasites

A direct action of GSH on parasitic organisms has been described by Datta and Banerjee (1988) who found that a concentration of 0.025 % GSH reduced the hatching activities of *Dysdereus* queens. Although the results support the possibility of a direct effect of GSH as a substance on the parasite, it remained questionable if *in vivo* exposure to this high GSH concentration is possible.

For fungal and bacterial pathogens, May et al. (1996b) could not find an altered response of GSH deficient plant mutants.

Glutathione is the cofactor needed in pests metabolism for pesticide resistance

Genuine or developed resistance of pests against pesticides is a great problem in plant protection. Glutathione has been found to play a key role in this matter and consequently related investigations evolved to become a major field of research.

Parasite endogenous glutathione (PEGSH) and the enzymes belonging to its metabolic pathways (especially glutathione *S*-transferases (GST)) are well known to play a role in the detoxification of a range of pesticides (e.g. parathione and its derivates) and thus are recognized as factors for the resistance of: a large variety of pests, mainly insects, and against various pesticides (Nomeir et al. 1987, Shivanandappa and Rajendran 1987, Kirby et al. 1994, Sun et al. 1990, Balabaskaran et al. 1989, Chiangh and Sun 1993, Ku et al. 1994, Suckling et al. 1990, Owusu and Korrike 1996, Sivori et al. 1997, Egaas et al. 1992, Gould and Hodgson 1980, Reidy et al. 1990, Yu 1996). Glutathione transferase is a collective term for the family of predominantly cytosolic isoenzymic proteins found throughout the animal kingdom (Reidy et al. 1990). They act by catalysing the conjugation of a broad range of compounds bearing an electrophilic site with reduced GSH (Reidy et al. 1990).

This mechanism is not only responsible for the detoxification of pesticides but also for many other xenobiotic compounds (Rose et al. 1989) and allelochemical stress in phytophagous parasites (Gunderson et al. 1986, Weinhold et al. 1990, Yu 1992). Glutathione *S*-transferase is known to be present in at least 24 insect families (Yu 1996). The activity of parasite endogenous GST again is influenced by the content of secondary metabolites which the parasite forages from its host. These compounds may stimulate or inhibit PEGST (Zhang et al. 1997, Zhang and Wong 1997, Lesyczynski et al. 1994, Lesyczynski and Dixon 1992). Examples are phenolic compounds in cereals, which are involved in resistance against aphids (Lesyczynski et al. 1994, Lesyczynski and Dixon 1992, Lee 1991). Soybean varieties with expressed resistance against soybean loopers have been found to have increased coumesterol concentrations, which Rose et al. (1989) identified as the reason for increased GST activities in the parasite.

Insects, for example *Drosophila spec.* (Cochrane et al. 1987) or *Plutella spec.* (Sun et al. 1990) or aphids (Lesyczynski et al. 1994) or *Trifolium spec.* (Reidy et al. 1990) with resistance against xenobiotic pesticides like malathion, organophosphorous derivatives or cyflurthrin detoxify the pesticides by GST activity and GSH is the cofactor in that enzymatic reaction.

Plant glutathione supports pesticide resistance of parasites

The sulphur status of a plant is a strong influencing factor for GSH concentration in its tissue (Schnug et al. 1995). Consequently higher GSH levels in plants devoured by parasites increase the pests vitality as observed in *Epilachna* species fed on soybean leaves with different SO_2 fumigations. From these results it has been concluded that air pollution improves the success of pests on crop plants (Hughes and Voland 1988). High amounts of S containing compounds devoured may also improve the vitality of the parasite. According to Capua et al. (1991) mites foraging on *Allium sativum,* which is well known for its high content of reduced S compounds (Haneklaus et al. 1997), had a higher GST activity. As a consequence of this also the rate of epoxidation (a detoxifying reaction involving GSH) of aldrin was also increased in the parasites.

Hughes et al. (1987) found also a better performance of *Trichoplusia spec.* on *Brassica* grown on sludges. As sludges are usually rich in sulphur, the sludge grown crops had higher GSH contents. This strengthens the hypothesis that pests benefit from higher GSH contents in their forage.

Host plants induce GST activity in the parasite (due to isothiocyanates and other allelochemicals) (Yu 1992). Yu (1982) showed that plants with

typically higher sulphur concentrations increase GST activity in *Spodoptera spec.* Consequently, highest in GST was *Brassica juncea* (*Brassica* species are among the plants with the highest sulphur concentrations) and lowest was *Cynodon dactylum.* Yu (1996) concluded from this that GST plays an important role in allelochemical resistance in phytophagous Lepidoptera. The same conclusions were drawn by Wadleigh and Yu (1987) who reported an important role of GST in isothiocyanate detoxification in phytophagous insects and proposed the involvement of GST in the food-plant adaptation of phytophagous insects.

Some authors could not find diet specific differences (e.g. Egaas et al. 1992), but this does not prove the null hypothesis, because the diet compared may be inadequate, for instance, when only diets of supposed same sulphur content are compared like in Egaas et al. (1992).

Finally it should be mentioned that obviously also varieties with higher resistance against an insect, induce higher GST activity in their predators, compared to a non-resistant variety (Lesyczynski et al. 1994).

INDIRECT INTERACTIONS BETWEEN GLUTATHIONE AND ANIMALS

The following physiological mechanisms involving GSH are called "indirect interactions" as they involve GSH as part of a physiological pathway but the result or end product does not consist of GSH.

Glutathione is part of a redox system affecting the reproduction of parasites

Miles and Oertli (1993) found that the infiltration of alfalfa stems with GSH and ascorbate reduced the reproduction rate of parasitic *Therioaphis spec.* and *Acyrthosiphon spec*. The authors proposed the hypothesis of a complex change in plant redox mechanisms involving GSH as responsible for aphid/plant interactions. This model is supported by El-Zahaby et al. (1995) who found that susceptible plant varieties seem to have a problem in an efficient recovery of oxidized ascorbate to ascorbic acid due to a lack of GSH.

Glutathione is part of the trigger mechanism for the recycling of sulphur from glucosinolates

Glutathione is involved in almost all biosynthetic pathways of secondary sulphur containing plant metabolites. Secondary sulphur containing plant metabolites again are well known for their significance in interactions between plants and other organisms (Schnug 1990, 1993, 1997). A well investigated example for this are glucosinolates, which attract insects (Staedler 1999), repel insects and other animals, and show antifungal and antimicrobial action like sinigrine (Schnug and Ceynowa 1990). In this particular case GSH is not directly involved in the biosynthesis of the glucosinolate structure but is part of a physiological mechanism, which regulates the glucosinolate concentration in the plant tissue by its catabolic rate. Glucosinolates are catabolized by the enzyme myrosinase yielding thiocyanates and sulphate (Schnug 1993) which again are recycled into the primary sulphur metabolism. This pathway has been identified as a vital storage strategy of glucosinolate containing plant species to adapt their high sulphur requirement to the ambient sulphur supply (Schnug 1988, 1993). The activity of myrosinase follows the ascorbate concentration with a pronounced optimum function and the ascorbate concentration is again dependent on the amount of GSH available to regenerate it from dehydroascorbate. Low sulphur supply causes low GSH concentrations in the plant, which again decreases ascorbate levels to the level where myrosinase activity reaches its maximum, yielding maximum rates of thiocyanates and sulphate released from glucosinolates. On the other hand, sufficient sulphur supply with higher GSH levels in the tissue regenerates ascorbate to levels at which myrosinase is virtually inactivated. With altered glucosinolate levels in the plant tissue their action on other organisms is changed also.

Environmentally influenced GSH levels in plants with secondary sulphur metabolites may therefore be indirectly involved in the expression of ecophysiological messenger substances or signals.

A far extended example for a signal in this context may be the phenomenon of white flowering in *Brassica* species (Schnug and Haneklaus 1995), which is induced by sulphur starvation causing the species specific involvement of GSH (see above). As a result of a lowered synthesis of proteins, sugars are enriched which again promote the formation of anthocyanins. In the flower petals anthocyanins occur as colourless leuco-anthocyanins which makes the petal turn from yellow to white and loosing its attractiveness for foraging honey bees (Schnug 1993).

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